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Progesterin and Antiprogesterin

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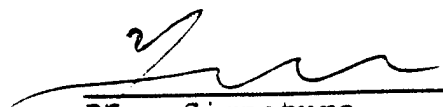
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## I. THE ORIGINAL HYPOTHESIS/PURPOSE

### Hypothesis

Growth of the normal mammary gland involves proliferation, differentiation, programmed cell death and remodeling of the basement membrane throughout the cyclic ovarian stimulation of the menstrual cycle and the pregnancy/lactation cycle. It now appears clear that progesterone, acting in a background of prior and concurrent estrogen exposure, is the principal effector of these three processes. One might expect that some aspects of these complex progestin-regulated events might be retained in breast cancer. Studies with human breast cancer cell lines in culture have provided clues that, like estrogens and antiestrogens, progestins and antiprogestins might also regulate both growth and metastasis. A major hurdle for fully testing this hypothesis has been the creation of human model systems whereby progestin- and antiprogestin-sensitive human cell lines are grown as tumors in the nude mouse. A second major hurdle has been no progestin sensitive breast cancer cell lines have been shown to be sufficiently metastatic in the nude mouse to allow testing of progestational and antiprogestational drugs that might interrupt this process. We have recently made progress in vitro and in development of in vivo models of metastatic breast cancer. Using our newly developed high PR expressing and metastatic TKS-7 (FGF-4 transfected T47D cells) and TLZ-5 (LacZ transfected T47Dco cells) breast cancer cells, as well as already established MKL-4 cells, we wish to continue these studies by testing the following hypothesis:

1. Metastasis as well as tumorigenesis of hormone-responsive human breast cancer cells can be regulated by progestins (such as MPA-medroxyprogesterone acetate); antiprogestins (such as ZK98,299-onapristone) can interfere.
2. Progestational and antiprogestational regulation of metastasis in our initial MCF-7 derived model (termed MKL-4) may relate to modification of angiogenesis, proteinase secretion, and adhesion.
3. Since a second breast cancer cell line T47D has more progesterone receptors than MCF-7 cells, development of a T47D metastatic model (TKS-7 and TLZ-5) may provide a system which is even more sensitive to progestational and antiprogestational regulation. In particular, tumor growth in addition to invasion/metastasis may be modulated.
4. Progestational and antiprogestational regulation of metastasis in TKS-7 and TLZ-5 models may relate to modification of non-intergrin 67kDa laminin receptor and our newly identified 80kDa matrix-degrading proteinase.

### Specific aims

We propose to continue our experiments *in vitro* and *in vivo*. We would like to continue our in vivo experiments with a complete analysis of antimetastatic effects of onapristone and potential promotional effects of MPA on MCF-7 and particularly our newly developed T47D models. It has taken several years to fully appreciate how to more completely adapt hormone dependent breast cancer lines for growth and metastasis in the nude mouse. We feel that we can now finally begin to evaluate the effects of hormonal (such as MPA) and anti-hormonal agents (such as onapristone) on both tumor growth and metastasis in responsive breast cancer cells. We will begin to address potential in vivo effects of MPA and onapristone action by evaluating their effects on tumor growth, angiogenesis and metastasis. We have established a collaboration with

Dr. Mark Sobel (NIH) to use his variety of agents for laminin receptor studies. Angiogenic studies will be in collaboration with Dr. Sandra Mcleskey (Lombardi Cancer Center, Georgetown, PI's former colleague) who has extensive experience with MKL-4 angiogenesis and metastasis. We have also discovered a new, broad substrates, basement membrane-, stromal matrix-degrading proteinase in hormone-responsive breast cancer cells [70]. We would like to study regulation of this proteinase, and other matrix-degrading proteinases by MPA and onapristone.

1. We propose to first carry out the time course of our initial studies of MPA and onapristone regulation of MKL-4 metastasis to more fully evaluate effects on metastasis.
2. We will develop T47D metastatic models (TKS-7 and TLZ-5). TKS-7 cells will be transfected with LacZ gene. We propose to test MPA and onaprostone treatments of LacZ transfected TKS-7 cells and TLZ-5 cells for tumorigenic and metastatic behavior.
3. We will test MPA- and onapristone-treated TKS-7 and TLZ-5 tumors for regulation of angiogenesis, proteinase levels including newly identified novel 80kDa proteinase, integrin levels and 67kDa laminin receptor levels. We will test MPA and onapristone treatments of TKS-7 and TLZ-5 cells *in vitro* for similar endpoints.
4. If progestational and antiprogestational effects are observed in our proposed T47D models, we will develop the tail vein model of experimental metastasis with two goals in mind to study the mechanisms underlying these regulations: 1) study of possible roles of 67kDa laminin receptor on the adhesion of tumor cells to the vascular endothelium and basement membrane during blood vessel extravasation and colonization; and 2) study of the biological relevance of the novel 80kDa matrix-degrading proteinase to these progestational and antiprogestational regulation of experimental metastasis.

## II. ADJUSTMENT

The current Innovative Development and Exploratory Award was initially awarded to study the effects of progestin **MPA** and antiprogestin **onapristone** on breast cancer invasion and metastasis. During the two-year period, we have made some adjustment for changing the scope based on following **rationales**:

1. A mainstay in the treatment of breast cancer has been tamoxifen. Although many tumors initially respond to tamoxifen, most ultimately relapse after several years and require second and third line therapies, such as other anti-hormonal agents. In this context, antiprogestins have emerged as potentially effective therapies. A new antiprogestin onapristone, manufactured by Schering AG Berlin, has been demonstrated to have antitumor activity in the hormone-dependent MXT mammary tumor model of the mouse and the DMBA- and the MNU-induced mammary tumor models of the rat (1). In an attempt to support the potential application of onapristone for breast cancer treatment, one of the goals of the proposed studies is to try to understand the mechanisms of action of onapristone in human breast cancer cells *in vitro* and *in vivo* in the nude mouse. However, the clinical trial for onapristone was terminated due to the toxicity. PI initially obtained onapristone from Dr. D. Henderson at Schering AG Berlin. Since Dr. Henderson has left the Schering AG Berlin and the company had no interest for the further study, the source of

onapristone was becoming problematic.

2. A recent clinical study has emphasized the potential importance of angiogenesis to metastatic spread of some human cancers and particularly in breast cancer (2-3). However, the role of FGF-4 on breast cancer progression has not been determined. PI realized that the FGF-4 transfected T47D cells (TKS-7) is an artificial model and may not represent or mirror clinical disease. A more relevant breast cancer related angiogenic factors (SF), such as scatter factor (4-6), should be used to generate T47D clones.

3. The estrogenic activity of synthetic progestins such as MPA has been reported (7-8). MPA can have dual effects through estrogen target tissues either to stimulate or differentiate cells (7). One report by M.H. Jeng and V.C. Jordan indicated that the estrogenic properties of MPA through estrogen receptor but not progesterone receptor is the growth-stimulatory mechanism (8). Based on these conflicting evidence regarding the both estrogenic and progestational activities of MPA, we concerned that our preliminary results on the stimulatory effect of MPA on TKS-7 cells could be mediated by estrogenic activity.

We have made the following changes:

1. Because of the proposed estrogenic activity of synthetic progestin MPA, we have used natural progesterone.

2. Establishment of relevant animal model systems is essential for progress toward understanding breast cancer biology and testing new therapies such as antiprogesterone agents. SF is a novel cytokine that stimulates tumor cell motility, invasion, and angiogenesis. Emerging evidences from our own and others suggest that SF may play a major role in breast cancer progression. We **hypothesize** that the development of a more malignant T47D model by SF cDNA mediated transfection will partially mimic breast cancer progression.

3. We and others have demonstrated that antiprogesterone treatment can induce growth inhibition and differentiation on breast cancer cells. However, little is known as to: a) genes regulated by progesterone; and b) the mechanisms underlying this growth inhibition. Peptides that locally signal growth cessation and stimulate differentiation of the developing epithelium is very important for mammary gland development and preventing cancer formation.

Antiprogesterone-induced growth inhibition on breast cancer cells is suggested to be mediated by antiprogesterone-induced functional differentiation. In an attempt to study the molecular pathways underline this antiprogesterone-induced mammary differentiation, we have turned our effort to the family of protein named as mammary derived growth inhibitor (MDGI). MDGI is a mammary epithelial cell growth inhibitor and differentiation factor. Studies of mouse and bovine mammary derived growth inhibitor (MDGI) suggest several potential functions of MDGI on growth and differentiation of mammary gland. Expression of MDGI in mouse mammary epithelial cells was increased during the functional differentiation induced by hormones such as prolactin and antiprogesterones; and this increased MDGI expression is associated with antiproliferative and differentiation promoting activities of antiprogesterones and prolactin (9-10). These data suggest a

local role of MDGI as a growth inhibitor in mediating or complementing hormonal action during differentiation. In this regard, MDGI is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells (11), which may be involved in the protective effect of early parity on a subsequent breast cancer incidence. So far, only bovine (12-13) and mouse (14-15) MDGI was identified and partially characterized. Using differential cDNA sequencing approach (16), we have identified a novel human tumor growth inhibitor for breast cancer cells (17-18). This novel tumor suppressing factor has some homology to mouse mammary derived growth inhibitor (MDGI) and thus was named "MDGI-related gene" (MRG).

- Task 1a. We did not carry out these experiments because of the rationales of 1-3.
- Task 1b. In a small pilot experiment, we injected  $0.5 \times 10^6$  TKS-7 cells into the tail vein of 10 nude mice. The animals were sacrificed at day 2, 4, 6, 8 and 10. The lungs of sacrificed animals were excised, fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin (H & E) for microscopic examination for morphologic evidence of tumor metastasis. Sections were reviewed and scored by pathologist. Breast cancer cells can be detected in the lungs of the mice sacrificed at day 2. No morphologic evidence of breast tumor cells was observed in the lungs from the mice sacrificed after two days of injection. We interpreted this data as a negative result. PI has discussed the results with some experienced investigators and share the similar comments regarding the tail vein experimental model: the tail vein approach suffers all of the criticisms as to its artificial nature and is not as meaningful.
- Task 2a Completed.
- Task 2b Completed.
- Task 2c Did not completed. See Task 1b for rationales.
- Tasks 3&4 Did not completed. See the rationales 1 and 3.

### III. BACKGROUND AND RATIONAL

#### 1. Progestational regulation of growth of mammary tumors

Mitogenesis and mutagenesis are major driving forces in neoplastic development. The major strategy of chemoprevention is to block the effects of both mutagens and mitogens. While little is known about important breast mutagens, much is known about breast cell mitogens. There is overwhelming epidemiologic evidence that breast cancer risk is closely related to overall exposure to steroid hormones. Regulation of growth and development of mammary gland involves a balance between the actions of the two major female sex steroid hormones, estradiol and progesterone. It is known that breast cancer is dependent upon estrogenic hormones in about one-third of clinical cases and can be inhibited by antiestrogenic antagonists (19). From an endocrinological aspect, the view that estrogen is the major adverse hormonal factor in onset and progression of human breast cancer has dominated thinking in this area (20). Conversely, the other ovarian steroid, progesterone, and its synthetic derivatives were initially thought to be protective, a view largely based on their differentiating and antiproliferative effects on endometrium (21-22). However There is a growing body of evidence suggests that the mechanisms by which estrogen and progesterone regulate the proliferation and differentiation of



uterine epithelial cells may not apply equally to the breast (23).

A. *In vitro* studies using established breast tumor cells. Evidence supporting the stimulatory effect of progestins on breast cancer cell growth includes its stimulatory effects on cell growth, expression of mitogenic growth factors and/or their receptors in steroid receptor-positive breast cancer cells:

- 1). *In vitro* studies using T47D cells, with progesterone or synthetic progestins have shown to stimulate cell growth (24-27)
- 2). Progestin induces expressions of several growth factors: EGF (28), TGF $\alpha$  (29), IGF-I (25), and IGF-II (30).
- 3). Progestin stimulates expression of growth factor receptors: EGF receptor (31), insulin receptor (27).
- 4). Progestin induces expression of cycle-related protooncogenes: c-fos and c-myc (32).
- 5). Blocking antibodies against TGF $\alpha$  (33) and IGF-I (26-27) are able to block or inhibit both progestin-stimulated colony formation and the increased colony formation induced by conditioned medium from progestin treated mammary tumors.

Although several lines of *in vitro* studies using established human breast cancer cells, as reviewed by Clarke and Sutherland (21), suggests that progesterone or synthetic progestins inhibit cell growth, there is no mechanistic correlation between this inhibitory effect on growth of breast cancer cells and the stimulatory effects of progestins on expression of mitogenic growth factors and/or their receptors in steroid receptor-positive breast cancer cells. Overall, progestin effects on cell proliferation are critically dependent on the precise combination of other steroids and growth factors present in the medium in which the cells are grown. In a rich medium, where growth is optimized, further growth enhancement is difficult to demonstrate, while inhibitory effect may be exaggerated. In a deprived medium the reverse is true. The lack of physiological consensus in these *in vitro* studies make these *in vitro* model invaluable for the analysis of molecular mechanisms underlying progestin effects on breast cancer cells.

B. The role of progesterone in an *in vivo* system. Recently, considerable evidence has suggested that progesterone may be more important than estrogen as an ovarian stimulus in driving proliferation of **normal** human and rodent breast epithelium. Studies in support of this come from both experimental models and normal cycling women:

- 1). Progesterone is more effective than estrogen in stimulation of proliferation of mouse mammary epithelium (34).
- 2). Stimulatory effects of progesterone on the development of mammary gland buds can be inhibited by progesterone antagonists (35).
- 3). It is notable that the major peak of estrogen levels occurs in the early menstrual cycle, when DNA synthetic activity is at a minimum, and in contrast, proliferation is optimal in the late menstrual cycle (days 20-28) in which the progestational level reaches the **peak** (36-37).
- 4). Studies of the mitotic rate during the normal menstrual cycle and in women taking oral contraceptives containing progestins further support the stimulatory effect of progestin on mammary epithelium (38).

Unlike estrogen, whose function as a major stimulus for onset and progression of **breast**

**cancer** is well established in women and experimental rodents, the role of progesterone is undefined. The original studies by Huggins (39), followed by the work of Robinson and Jordan (40) and Grubbs et. al. (41) clearly show that ovarian progesterone is not likely to be inactive or protective in carcinogenesis. A progestational environment increases tumor incidence (34,42). The endocrine requirements of carcinogen-induced mammary tumors are complex and vary according to species and whether the manipulations are carried out before or after carcinogen administration. Interestingly, progesterone has been reported to both stimulate and inhibit the growth of experimental tumors depending upon the dose and the experimental preparations. Although progesterone treatment several weeks prior to a carcinogen protects against breast cancer, progesterone is a fully active tumor promoter if applied during or after carcinogen treatment (43). Thus, in relation to the human situation, these **original** animal data can be used to support either model depending on whether one is postulating effects before or after tumor initiation.

In **summary** of these recent *in vitro* and *in vivo* studies suggest that progesterone may be more important than estrogen as an ovarian stimulus in driving proliferation of normal human and rodent breast epithelium (44). One might expect that some aspects of these complex progestin-regulated events might be retained in breast cancers. In fact, the more recent experiments have demonstrated that progestational hormones stimulate the growth of carcinogen-induced and transplantable rat mammary tumors (45-46), and spontaneous mouse mammary tumors (47). These observations could explain the increasing number of reports connecting the pill use in young women with increased risk of breast cancer, and particular the very recent report on the New England Journal of Medicine showing that the risk of breast cancer was significantly increased among women who were currently using estrogen alone or estrogen plus progestin or progestin alone as compared with postmenopausal women who had never used hormones (48). Therefore, progesterone has been suggested as a stimulus for the growth of human breast tumors. A major hurdle for fully testing this hypothesis has been the creation of human model systems whereby progestin-sensitive human cell lines are grown as tumors in the nude mouse. We are currently proposing to develop a T47D-derived and progestin-sensitive model(s) for evaluating progestational and antiprogestational regulation of breast cancer growth and progression.

## **2. Progestational regulation of invasion-related genes.**

**A. Stimulation of 67kDa laminin receptor (67kDa LR).** Expression of 67kDa LR is associated with metastasis of colon (49) and breast cancer (50-51). Earlier work had demonstrated that the laminin binding capacity of plasma membranes isolated from human invasive breast carcinomas is 50-fold greater than that of membranes from benign lesions or normal tissue (51). We (52-53) and others (54) have observed that treatment of T47D cells with progestin R5020 and MPA results in a considerable increase in the expression of 67kDa LR protein and that this increased expression correlates very well with an increase in laminin attachment.

**B. Regulation of TIMP-2.** Progestin, but not estrogen, has been demonstrated to have ability to down-regulate TIMP-2 expression in hormone-responsive MCF-7 and T47D cells (55). It is anticipated that such down-regulation of TIMP-2 may increase the metastatic potentials.

## **3. Antiprogestins as new therapeutic tools for treatment of breast cancer**

A mainstay in the treatment of breast cancer has been tamoxifen. Although many tumors initially respond to tamoxifen, most ultimately relapse after several years and require second and third line therapies, such as other anti-hormonal agents. In this context, antiprogestins have

emerged as potentially effective therapies. Currently only two small clinical trials have been reported. The results from the first trial (56) indicates that RU486 at 200 mg/day led to partial regression in 12 of 22 (53%) women after 4 to 6 weeks of treatments. The second trial conducted involved 11 postmenopausal patients with metastatic breast cancer. RU486 was used as second-line therapy after first-line treatments with tamoxifen. Six of 11 patients showed a short-term (3 to 8 months) stabilization of disease, and one had an objective response lasting 5 months (57). Recently, effect of antiprogestins on the experimental mammary carcinogenesis has been addressed (58-59). Onapristone has been demonstrated to have antitumor activity in the hormone-dependent MXT mammary tumor model of the mouse and the DMBA- and the MNU-induced mammary tumor models of the rat (59). In addition, some remissions were seen in phase I studies with advanced disease in breast cancer patients. Although early studies suggest that the antitumor mechanism of these progesterone antagonists is a receptor mediated antiproliferative effect by induction programmed cell death (58), its mechanism(s) of action on human breast cancer are not yet known. One of the goals of the proposed studies is to attempt to more clearly understand the mechanisms of action of the antiprogestin onapristone in human breast cancer cells *in vitro* and *in vivo* in the nude mouse.

#### **4. Scatter Factor (SF) in breast cancer progression**

SF [also known as hepatocyte growth factor (HGF)] is a novel cytokine/growth factor that simulates tumor cell motility, invasiveness, and angiogenesis (59-62). Its receptor is a transmembrane tyrosine kinase encoded by a proto-oncogene (c-met) (63). Several findings by ourselves and other investigators suggest that SF may play a role in breast cancer progression.

- 1) SF induces cell motility and invasion in a variety of human cancer cell lines, including breast cancer (60).
- 2) SF is an extremely potent inducer of angiogenesis, in nanogram quantities (61-62).
- 3) SF and the c-met receptor are expressed *in vivo* in human breast cancers, with the highest levels of expression in the most aggressive cancers (4,6).
- 4) Treatment of EMT6 mouse mammary tumor cells *in vitro* with SF enhances their invasive phenotype and increases their ability to form lung colonies following intravenous injection into isogeneic hist mice (64).
- 5) SF-transfected MDA-MB-231 human breast cancer cells implanted in the mammary fat pads of nude mice exhibit significantly increased tumor growth rates as compared with control transfected cells, due, in part, to increased tumor angiogenesis (5)

#### **5. Mammary derived growth inhibitor (MDGI)**

A. MDGI in mammary gland. MDGI is a mammary epithelial cell growth inhibitor and differentiation factor firstly purified from the lactating bovine mammary gland (65) and then from cows milk (66). It belongs to a multigene family of conservative proteins, referred to as fatty acid binding proteins (FABP) (67). Based on mouse and bovine MDGI and mouse model, several lines of functions of MDGI on growth and differentiation of mammary gland have been demonstrated:

- 1). The growth and differentiation regulating properties of MDGI are reported. Recombinant and wild-type forms of MDGI specifically inhibit growth of normal mouse mammary epithelial cells (MEC) and promote morphological differentiation, stimulate its own expression and promote milk protein synthesis (68).
- 2). Selective inhibition of endogenous MDGI expression in MEC by antisense phosphorothioate oligonucleotides suppresses alveolar budding and impairs  $\beta$ -casein

- synthesis in organ cultures (68).
- 3). MDGI is also demonstrated to function as a potent tumor suppressor gene. Human breast cancer cells transfected with bovine MDGI exhibited differentiated morphology, reduced proliferation rates, reduced clonogenicity in soft agar, and reduced tumorigenicity in nude mice relative to control (69).
  - 4). Human homologue of bovine MDGI has been mapped to chromosome 1p33-35 (69), a locus previously shown to exhibit frequent loss of heterozygosity in human breast cancer (70-71).
  - 5). Expression of MDGI in mouse mammary epithelial cells was increased during the functional differentiation induced by hormones such as prolactin and antiprogestins; and this increased MDGI expression is associated with antiproliferative and differentiation promoting activities of antiprogestins and prolactin (9-10).
  - 6). MDGI also suppresses the mitogenic effects of epidermal growth factor (EGF), and EGF antagonizes the activities of MDGI (72).

#### IV. WORK ACCOMPLISHED

##### 1. New metastatic breast cancer cell lines.

We have focussed on preexisting breast cancer cell lines MCF-7 and T47D for our studies because of their positivity for receptors for estrogen and progesterone. Although MCF-7 and T47D were derived from a highly metastatic tumor, the properties of these cell lines in vivo in the nude mouse have been disappointing; only poorly metastatic, encapsulated tumors are generally observed. This poor metastatic potential may be due to the low degree of tumor vascularization. For these reasons, the gene for the angiogenic growth factor FGF-4 and SF have been transfected into cells. The chromogenic enzyme LacZ has also been transfected into MCF-7 cells. This facilitates the detection of micro metastases resulting from cells acquiring the ability to convert the chromogenic substrate X-gal to blue color, which is easily observed under a dissecting microscope. We will use **three series** of cells for study hormonal regulation of breast cancer growth and metastasis in nude mice (Table 1):

**Table 1**  
Nomenclature of cell lines

Name	Characteristics
MCF-7 mouse	Hormone responsive, estrogen-dependent tumorigenic, non-metastatic in the nude mouse
MKL-4	Clone of MCF-7 transfected with FGF-4 and LacZ, highly tumorigenic and metastatic; micro-metastasis can be visualized by incubation of organs with chromogenic substrate X-gal.
ML-20	Clone of MCF-7 transfected with LacZ gene, with identical properties to parental.
T47D	Hormone responsive, expression of high levels of progesterone receptor, poorly tumorigenic in the nude mouse.
TKS-7	Clone of T47D transfected with FGF-4 expression vector; highly tumorigenic in the nude mouse; massive metastases in lymph node were observed.
SF-T47D	SF transfected T47D cells. Need to be characterized.

Among these cell lines with the metastatic potentials, the **first** series of MKL-4 cells were established in Drs. Kern and Mcleskey's lab at Lombardi Cancer Research Center, Georgetown University where the PI was a postdoctoral fellow. The MKL-4 cells were initially chosen in PI's lab to study the effects of estrogen, antiestrogen tamoxifen and ICI on regulation of micrometastasis.

The **second** series derived from T47D cells. Since the MCF-7 cells are traditionally estrogen sensitive human breast cancer cells, and may not be a valuable model for study progestin effects due to the very low levels of progesterone receptor (PR), we are interested in developing new metastatic T47D derived models. As previously demonstrated, hormone responsive metastatic MCF-7 cells can be obtained by utilizing cDNA-mediated transfection of an angiogenic growth factor, FGF-4 (73-74), we therefore have adapted the same approach to T47D cells. For that, T47D cells were transfected with the same FGF-4 vector as MCF-7 cells (75-76). To detect biologically active FGF-4 secretion by TKS cells, a cross-feeding experiment using SW-13 adrenal carcinoma cells were performed as described before (73). As demonstrated in **Table 2**, clone TKS-7 expressed higher levels of FGF-4 than that of TKS-2 cells. The secreted levels of FGF-4 from TKS-7 cells is compatible to previously established metastatic MKL-4 cells.

**Table 2**  
Detection of secreted FGF-4 by transfected T47D clones

Conditioned medium derived from cell line	Colonies of FGF-4-sensitive SW-13 cells (x 10 <sup>3</sup> )
Cell-free control	4±1
T47D	10±3
TKS-2	32±11
TKS-7	90±21
MKL-4	85±13

T47D cells were transfected by FGF-4 as previously described for MCF-7 cells [6]. A bioassay for FGF-4 secretion using cloning of SE-13 cells as previously described [6] was next carried out. A high number of SW-13 colonies indicates high secretion of a FGF-related molecule. Numbers are mean plus SD of triplicate determination.

We have begun to test the *in vivo* behavior of TKS-7 cells. The results in **Fig. 1** show that the tumor, following 6 weeks treatments with tamoxifen and progesterone, was very aggressive, sometimes quite bloody (**Fig. 1A, left mouse**), and highly vascularized (**Fig. 1B**). In addition, the massive lymph node metastases were also observed (**Fig. 1C**). Therefore the TKS-7 model appears to be suitable for study the effects of progestational hormones and antiprogestins in breast cancer growth and metastasis because of its metastatic potential and particularly expression of high levels of PR.

The **third** series are currently under investigation. In an attempt to develop a more clinically relevant breast cancer progression model, we have transfect T47D cells with SF. SF is a novel cytokine/growth factor that stimulates tumor cell motility, invasiveness, angiogenesis, and its expression is associated with breast cancer malignant progression as we have already discussed.

## **2. Effects of progestational hormones and antiprogesterin on TKS-7 tumor growth**

**Progestin MPA.** As demonstrated in **Fig. 2 & 3** from two experiments, TKS-7 behaviors like a hormone-independent but still hormone responsive cell line. While estrogen is still a major driving force for TKS-7 tumor growth, inducing a 16-fold increase over control and a 100% tumor incidence, progestin MPA stimulates the tumorigenesis 2.5 fold over control; the tumor incidence was increased from 50% of control group to 90% of MPA-treated group (**Fig. 2**). Vector alone transfected parental T47D cells were also examined for their responses to hormones. As we previously reported (53), parental T47D cells were poorly tumorigenic in both control and MPA-treated mice, no T47D tumors were observed. In mice implanted with the estradiol pellets, T47D cells formed small non-progressive tumors that subsequently regressed after 4 weeks (data not shown).

**Progesterone.** Several reports have demonstrated that synthetic progestins exert some estrogenic activity (7-8). This raises the concern that the MPA-induced TKS-7 tumor growth may be mediated by a weak estrogenic activity but not by progestational activity. In order to rule out this possibility, we have begun to use progesterone instead of progestin. As we anticipated, like MPA, progesterone stimulates the tumorigenesis 5 fold over control; the tumor incidence was also increased from 50% of control group to 70% of progesterone-treated group (**Fig. 3**).

**Antiprogesterin onapristone.** We have obtained a newly developed antiprogesterin onapristone from Dr. Henderson at Schering AG Berlin. This new antiprogesterin onapristone (ZK98,299), a derivative of RU486, has high antiprogesterin activity but low antiglucocorticoid activity (77). Because of its antitumor activity in experimental mammary tumor models, we have explored its potential therapeutic value against breast cancer in our TKS-7 hormone-responsive human breast tumor model. As demonstrated in **Fig. 3**, onapristone alone did not change the basal levels of tumorigenic activities of TKS-7 cells as measured by tumor size and tumor incidence. However, when the antiprogesterin pellet was inoculated to progesterone-treated mice, onapristone significantly inhibits the progesterone-induced tumor growth. The tumor size was reduced from 210 mm<sup>3</sup> in progesterone-treated group to 77 mm<sup>3</sup> in progesterone- and onapristone-treated group (64% inhibition); no significant change of tumor incidence was observed. Lymph node metastasis were also examined by histological analysis. No lymph node metastasis was observed in control mice. However, we identified four lymph node metastases in progesterone-treated mice. Furthermore, onapristone treatment resulted in a reduction of progesterone-induced lymph node metastasis (**Fig. 3**). These results are consistent with the previously established metastatic MCF-7 cells showing that the rate of metastasis depends upon time since inoculation and tumor size (73-74).

**Progesterone and tamoxifen.** We are also interested in the effects of antiprogesterin alone compared to its effects in combination with antiestrogen in an attempt to test the new endocrine therapy in the tamoxifen relapse setting. The rationale behind this antiestrogen-antiprogesterin interaction derives from our assumption that antiestrogen tamoxifen, also a partial estrogen agonist, may increase PR expression in breast cancer cells and therefore enhance their responses to progesterone and onapristone. As demonstrated in **Fig. 4**, a **synergistic** effects of tamoxifen and progesterone was observed in TKS-7 tumor growth. Progesterone alone stimulated tumor growth 4-fold over control; tamoxifen also stimulated tumor growth (2.5-fold), although to less extent as compared to progesterone, presumably due to its estrogenic activity. When the mice were treated with a combination of tamoxifen and progesterone, the resulting TKS-7 tumors were significantly larger than that of tamoxifen alone or progesterone alone. The tumor size was

increased from 83.6 mm<sup>3</sup> in tamoxifen-treated group and 134 mm<sup>3</sup> in progesterone-treated group to 259 mm<sup>3</sup> in the combination group. Picture in **Fig. 5** shows the tumors from control, progesterone-treated, tamoxifen-treated and progesterone/tamoxifen-treated mice. Such synergistic interaction between tamoxifen and progesterone on TKS-7 tumor growth may due to the tamoxifen-mediated estrogenic effect on up-regulation of PR. We anticipate that the PR levels in tamoxifen-treated mice are increased as compared to that of non-treated or progesterone-treated mice.

### **3. Antitumor activity of the novel human breast cancer growth inhibitor MRG**

**Molecular cloning of MRG cDNA.** We generated cDNA libraries from breast cancer biopsy specimen and normal breast and analyzed these libraries by EST-based differential cDNA sequencing. ESTs were sequence analyzed and grouped to different groups based on sequence overlapping, and each unique EST group was first analyzed for relative expression in the cDNA libraries from normal breast versus breast cancer and then subjected to tissue-specific expression by examining tissue origins of individual EST sequences against approximately one million of ESTs derived from a variety of different tissue types. As previously demonstrated (16), we identified three classes of EST groups that were differentially expressed in normal breast versus breast cancer: 1) genes more abundant in breast cancer than in normal breast; 2) genes that are more abundant in normal breast than in breast cancer; and 3) genes that are selectively expressed in breast relative to other tissue types. Within the second class, the automated screening revealed a group of 25 ESTs encoding a novel gene with homology to mouse MDGI (greater than 30% homology). Of the 25 distinctive MDGI-related EST clones, 4 ESTs were derived from normal breast library, and the rest of ESTs were derived from fetal brain library. No ESTs were derived from breast cancer library. After sequencing of these cDNA fragments, one clone containing a start codon (ATG) and an open reading frame was isolated and characterized.

The nucleotide sequence determined from this clone and the predicted corresponding amino acid sequences are shown in **Fig. 6**. The full-length cDNA sequence contains 731 bp with 396 bp open reading frame; 75 bp in the 5' untranslated region; and 260 bp of 3' untranslated sequence. The open reading frame extends from the initiation A<sub>76</sub>TG codon to TAA<sub>474</sub> stop codon. The open reading frame encodes a protein of 132 amino acids. The deduced amino acid sequence predicts a protein with an isoelectric point of 5.4. Comparisons of the predicted amino acid sequence with the sequence of similar human proteins are shown in **Fig. 7**. After optimal alignment, the putative protein shows 63.4% amino acid identity to mouse MDGI, 68.9% identity to bovine MDGI, 66.2 % identify to heart derived fatty acid binding protein (H-FABP), and 56.8% identity to adipocyte derived fatty acid binding protein (A-FABP). The extensive similarity of the predicted amino acid sequence with MDGI suggests that the putative new protein is a MDGI-related and therefore this gene is designated as MRG.

**Expression of MRG in human breast epithelial cells.** Since the differential cDNA sequencing revealed MRG expression in normal breast library but not in breast cancer library and its sequence homology to MDGI, a previously described bovine- and mouse-derived growth inhibitor for mammary epithelial cells, we speculate that MRG is a potential tumor suppressor gene or growth inhibitor for mammary gland. In an attempt to evaluate the potential biological significance of MRG on human breast cancer development and progression, we first studied MRG gene expression in human biopsy samples from breast carcinoma and normal breast tissue.

Northern blot analysis (Fig. 8A) allows the detection of MRG expression in all four normal breast reduction mammoplasty specimens, but not in two malignant breast cancer samples. The presence of MRG in normal breast and the loss of MRG expression in breast carcinomas suggest a possible role of down-regulation of MRG in the development of breast cancer. The expression of MRG was also investigated in some human breast cancer cell lines (Fig. 8B). No expression of MRG was detected in any of the tested human breast cancer cell lines except T47D cells.

In order to localize the cellular source of MRG expression and to further assess the biological relevance of the loss of MRG expression in breast cancers, we next performed *in situ* hybridization on fixed sections from a variety of different human breast specimens. In these experiments, we examined two aspects of MRG expression, including the tissue localization (stromal versus epithelial) and the correlation of the loss of MRG expression and breast cancer malignant phenotype. Fig. 9 shows a representative *in situ* hybridization for MRG. We found a strongly positive MRG hybridization in epithelial cells of normal mammary gland (Fig. 9A & B) and benign fibroadenoma (Fig. 9C). The expression of MRG mRNA was detectable in the epithelial cells in all 5 reduction mammoplasty specimens and in 5 benign fibroadenomas. In contrast, expression of MRG was absent in 9 out of 10 cases of infiltrating breast carcinomas and in 10 out of 12 ductal carcinoma *in situ* (DCIS) samples. A representative negative staining of MRG in DCIS neoplastic epithelial cells (Fig. 9E) and in an infiltrating breast carcinoma (Fig. 9F) are presented. These *in situ* hybridization results are consistent with the Northern blot analysis which showed MRG expression in normal breast but not in breast carcinomas.

It is interesting to note that although a strong MRG signal was easily detected in the breast epithelial cells of normal gland and benign fibroadenomas, the benign breast hyperplasia showed different MRG expression patterns. Among 10 benign hyperplasias, 4 specimens were negative and 6 specimens were lightly stained for MRG expression. As illustrated in Fig. 9D, the intensity of MRG staining in a benign hyperplasia was greatly reduced compared to normal mammary gland (Fig. 9A & B) or benign fibroadenoma (Fig. 9C). The decreased MRG expressions in hyperplasia and the loss of MRG expression in breast carcinoma suggest that expression of MRG is stage-specific in breast: down-regulated during the proliferative stage and lost in breast cancer.

Tissue expression. Tissue-specific transcription of MRG was examined by Northern blot on 2 µg of poly (A) RNAs from various human adult tissues (Fig. 10). As expected, the Northern blot showed that MRG was abundantly expressed as a 1.1- kb transcript in brain, in which 21 MDGI-related ESTs were identified, and in heart and skeletal muscle which are the rich source for H-FABP. A 2.2 kb transcript with much lower accumulations in their relative intensity was also detected in heart, skeletal muscle, and pancreas. By contrast, none of them was present in other specimens analyzed, such as placenta, lung, liver, kidney, spleen, thymus, prostate, testis, uterus, colon, and small intestine.

Transfection and selection of MRG positive clones. Since we demonstrated a loss of MRG expression in breast cancer compared with normal or benign breast, we next asked if we could suppress breast cancer growth by overexpression of MRG gene in breast cancer cells. We selected MDA-MB-231 cell line as recipient for MRG mediated gene transfection because of 1) its lack of detectable MRG transcript; and 2) more aggressive and highly tumorigenic behavior in nude mice. Cells were transfected with a plasmid vector containing a neomycin resistance gene (neo-231 clones), or the same vector containing a full-length MRG cDNA (MRG-231 clones). Clones were initially screened by *in situ* hybridization on slides with a specific MRG antisense probe (data not shown), and the positive clones were subjected to Northern blot analysis. Six MRG-231 clones were picked up by *in situ* hybridization. Fig 11A. shows the Northern blot analysis of



MRG expression in selected clones. All two selected MRG-231 clones MRG-231-6 and MRG-231-10 expressed MRG mRNA transcripts. In contrast, none of the parental MDA-MB-231 cells or neo-231-1 and neo-231-2 clones produced any detectable MRG transcripts.

Effect of MRG transfection on cell growth. The growth curve of MRG-231 cells was compared to that of MRG negative MDA-MB-231 and neo-231-1 cells in a monolayer culture (**Fig. 11B**). Cell growth was inhibited approximately 65-75% ( $p < 0.001$  by Student's *t*-test) in the MRG-231-6 and MRG-231-10 cells compared to the parental MDA-MB-231 and neo-231-1 cells. There was no significant difference in the growth pattern between parental MDA-MB-231 cells and neo-231-1 cells.

Suppression of tumorigenicity by MRG transfection. To study the effect of MRG expression on tumorigenicity, The tumorigenicities of MRG-231 clones were determined in comparison with parental MDA-MB-231 cells and neo-231 clones in an orthotopic nude mouse model. Two independent experiments were done to confirm reproducibility and the data were shown in **Table 3**. After a lag phase of approximately 10 days, mice given implants of both MRG positive and MRG negative cells developed tumors. There was no significant difference in tumor incidence among the groups. Starting at about 30 days after inoculation, great level of tumor necrosis was observed in tumors derived from MDA-MB-231, neo-231-1, and neo-231-2 cells. The same breast cancer cells transfected with MRG, however, were significantly inhibited in their tumor growth; and either no or low level of tumor necrosis was observed. The mean volume of MRG-231-6 and MRG-231-10 tumors was only 21% of that in parental MDA-MB-231 tumors, 24% of that in neo-231-1 tumors, and 18% of that in neo-231-2 tumors, respectively ( $P < 0.005$  by Student's *t* test). **Fig. 11C** shows growth kinetics of one representative experiment from parental MDA-MB-231, neo-231-1, neo-231-2, MRG-231-6, and MRG-231-10 tumors. After a slow growth phase of 25 days, tumors from both parental MDA-MB-231 cells and two neo-MDA-231 clones increased in volume at an exponential rate. In contrast, the growths of MRG-231-6 and MRG-231-10 cells were dramatically inhibited. Thus, the tumorigenicity of the human breast cancer cells was significantly inhibited by expression of MRG.

Inhibition of the growth of breast cancer cells by recombinant MRG protein (MRGP). Previous studies have demonstrated that MDGI derived from the artificial bovine MDGI cDNA specifically inhibited the growth of normal mouse mammary epithelial cells (MEC). To test whether MRG can function like a growth inhibitor for human breast cancer cells, we generated recombinant MRG protein (MRGP) in baculovirus-infected cells. As demonstrated in **Fig. 12A**, MRGP treatment induced a dose-dependent growth inhibition in MDA-MB-231 and MDA-MB-435 breast cancer cells. Treatment of MDA-MB-231 cells with 9 nM MRGP resulted in 20% growth inhibition, whereas no growth inhibition was observed for MDA-MD-435 cells. At 132 nM of MRGP, the growth rates of MDA-MB-231 and MDA-MB-435 cells were inhibited about 50%. Interestingly, the growth of MCF-7 cells was resistant to the MRGP treatment. In contrast to previously described MDGI, the growth inhibitory activity of MRGP is not limited to mammary cells. As shown in **Fig. 12B**, the growth of TSU and Du-145 prostate cancer cells were also inhibited by MRGP in a similar dose-dependent manner. The growth of cervical cancer cell line C33A was slightly inhibited. MRGP had no effect on fibroblasts and microvessel endothelial cells (**Fig. 12C**). Interestingly, MRGP did not affect the growth of normal human mammary epithelial cells.

**Table 3.** Effects of MRG expression on tumor incidences and sizes of MDA-MB-231 cells

Experiment	Treatment Group	Tumor Incidence Tumor/Total (%)	Tumor Vol (mm <sup>3</sup> ) of Primary Size
1	MDA-MB-231	9/10 (90)	679 ± 169
	neo-231-1	8/10 (80)	704 ± 62
	neo-231-2	8/10 (80)	792 ± 59
	MRG-231-6	9/10 (90)	151 ± 29
	MRG-231-10	8/10 (80)	64 ± 17
2	MDA-MB-231	10/10 (100)	824 ± 215
	neo-231-1	9/10 (90)	645 ± 157
	neo-231-2	9/10 (90)	957 ± 182
	MRG-231-6	8/10 (80)	212 ± 41
	MRG-231-10	9/10 (90)	179 ± 53

Four hundred thousand of the cells were injected at day one into the mammary fat pads, and tumor volumes were determined as described in Materials and Methods. Volumes are expressed as means ± SEs (number of tumors assayed). For both experiments, total 10 injections were given to 5 mice in each group (two injection per mouse), and the mice were sacrificed 40 days after injection. Statistical comparison for primary tumors was analyzed by Student's *t* test. Statistical analysis for pooled MRG positive clones relative to pooled MRG negative clones gave  $P < 0.005$  for all the following comparisons: MRG-231-6 vs. MDA-MB-231; vs. neo-231-1; vs. neo-231-2, and MRG-231-10 vs. MDA-MB-231; vs. neo-231-1; and vs. neo-231-2.

## V. CONCLUSIONS

Growth of the normal mammary gland involves proliferation, differentiation, programmed cell death and remodeling of the basement membrane throughout the cyclic ovarian stimulation of the menstrual cycle and the pregnancy/lactation cycle. It now appears clear that progesterone, acting in a background of prior and concurrent estrogen exposure, is the principle effector of these processes. One might expect that some aspects of these complex progestin-regulated events might be retained in breast cancer. Using our genetically modified T47D model, we have demonstrated:

- 1). Tumorigenesis as well as metastasis of hormone-responsive human breast cancer cells can be stimulated by progestational hormones and antiprogestins (such as onapristone) can interfere.
- 2). Antiestrogen tamoxifen may potentiate antiprogestin onapristone-induced tumor regression due to its up-regulation of PR levels.
- 3). We identified and cloned, by using expressed sequence tag-based differential cDNA sequencing approach, a novel human antitumor growth inhibitor whose product exhibits some homology to mouse mammary derived growth inhibitor (MDGI) and thus named as MDGI-related gene (MRG).

4. Expression of MRG is lost during breast cancer malignant progression. *In situ* hybridization has detected MRG in normal and benign mammary epithelial cells but not in malignant breast cancer cells.
5. Transfection of MRG cDNA to human breast cancer cells dramatically suppressed tumor cell growth *in vitro* and *in vivo* in an orthotopic nude mouse model.
6. Purified recombinant MRG protein (MRGP) inhibited the growth of human breast cancer cells. In addition, MRGP also inhibited the growth of human prostate and cervical cancer cells. No effect of MRGP was observed in fibroblast and endothelial cells. Interestingly, MRGP had no effect on normal mammary epithelial cells.

MRG revealed no sequence homology to any of the hitherto known growth inhibitors except partial homology to mouse and bovine MDGI. Until now, only bovine and mouse MDGI have been identified and characterized. Studies of mouse and bovine MDGI suggest several functions of MDGI on growth and differentiation of mammary gland. The magnitude of the tumor suppressing activity of MRG is comparable to that previously observed for *Rb* and *p53*. The loss of MRG expression in malignant breast cancers and the inhibition of breast tumor growth by re-expression of MRG suggests that MRG is one of the growth inhibitors that locally signal growth cessation of the mammary gland. In fact, the human homologue of bovine MDGI has been mapped to chromosome 1p32-35, a locus previously shown to exhibit frequent loss of heterozygosity in human sporadic breast cancer. Therefore, the loss or down-regulation of MRG or its receptor may lead to abnormal growth and the development of breast cancer. The scenario that progesterone may adversely influence the onset and progression of breast cancers, as we demonstrated in this report, suggests that progesterone antagonists could play a role on the control of hormone-dependent breast cancers. Therefore, blockade of endogenous progesterone with antiprogestins would seem to be an important therapeutic goal. In fact, we demonstrated an inhibitory activity of antiprogestin onapristone on breast tumor growth; and this growth inhibition may be mediated by induction of MRG expression, and therefore blocking MRG expression may suppress the antiprogestin-induced growth inhibition and differentiation.

No graduate students have participated in this project. Therefore, this award did not result in a graduate degree.

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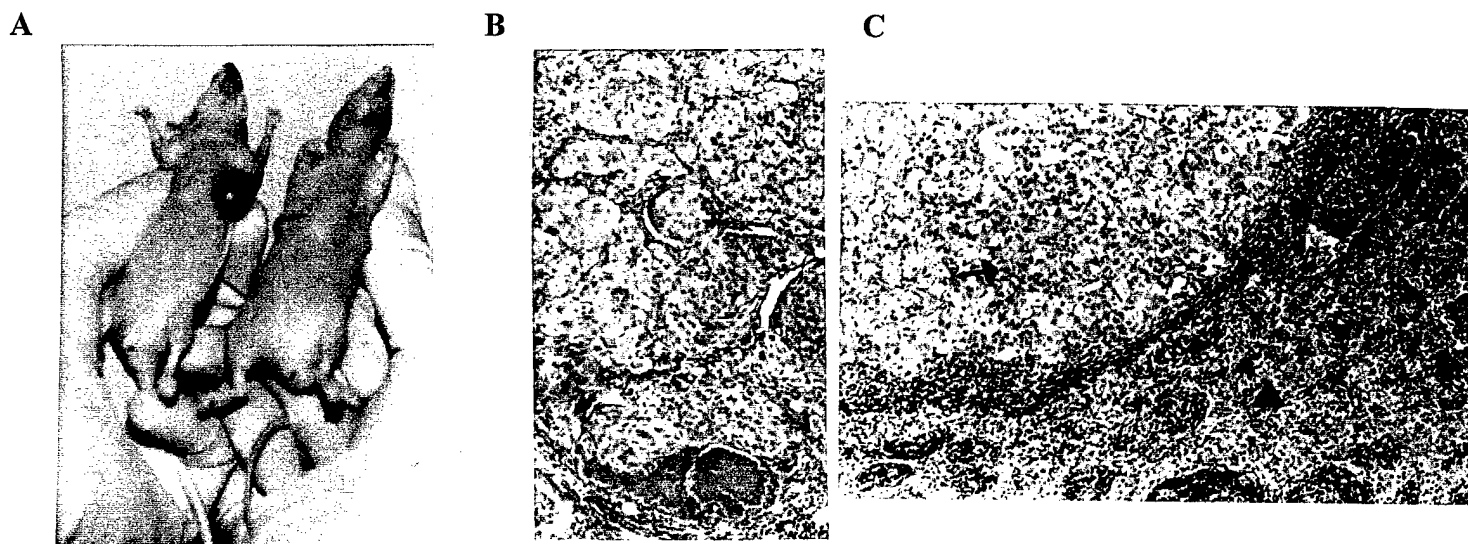
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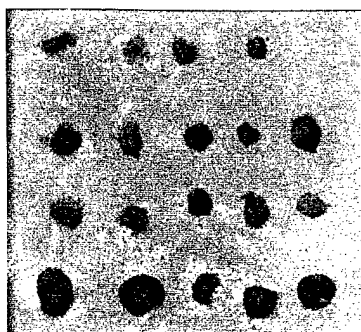
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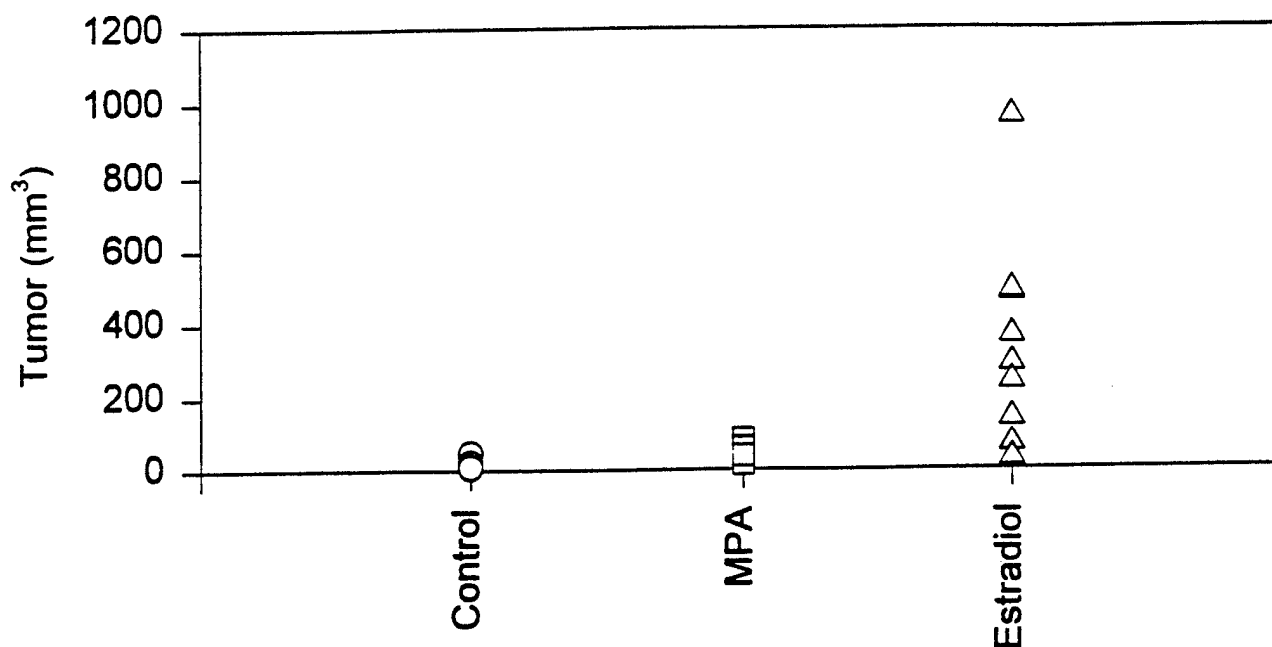
**Fig. 1.** Growth of TKS-7 cells in nude mice. 5 millions cells were injected into the mammary fat pad of the ovariectomized mice. Animals were scarified 6 weeks after inoculation, and the tumors and lymph nodes were processed for analysis. **(A)** Nude mice bearing TKS-7 tumors. Right mouse was treated with slowly time-releasing pellets of tamoxifen (5 mg/60 days) and progesterone (10 mg/21 days); left mouse was non-treated control. **(B)** Evolution of histology of very vascularized tumors from tamoxifen- and progesterone-treated mouse. **(C)** Evolution of histology of ipsilateral lymph node metastasis from tamoxifen- and progesterone-treated tumor.



**Fig. 5.** Regulation of TKS-7 tumor growth by steroid hormones. 5 millions cells were injected into the mammary fat pad of the ovariectomized nude mice. The mice were divided into four groups: control (first line); progesterone-treated, 10 mg/pellet/21 days (second line); tamoxifen-treated, 5 mg/pellet/60 days (third line); and progesterone- and tamoxifen-treated (fourth line). Tumors were harvested after 6 weeks. 5 representing tumors from treated groups and 4 representing tumors from control group were placed on the dryice. Although both progesterone and tomoxifen stimulated tumor growth, combined treatment gave the biggest stimulation. Please **notice** that the differences of the real tumor sizes among the different groups are more dramatic than it looks like in the picture because the tumor sizes in the picture only reflect the 2-D measurement. Fig. 4 and Table 4 (p20) also demonstrate such differences.



**Fig. 2 . Effects of MPA and estradiol on TKS-7 tumor growth**

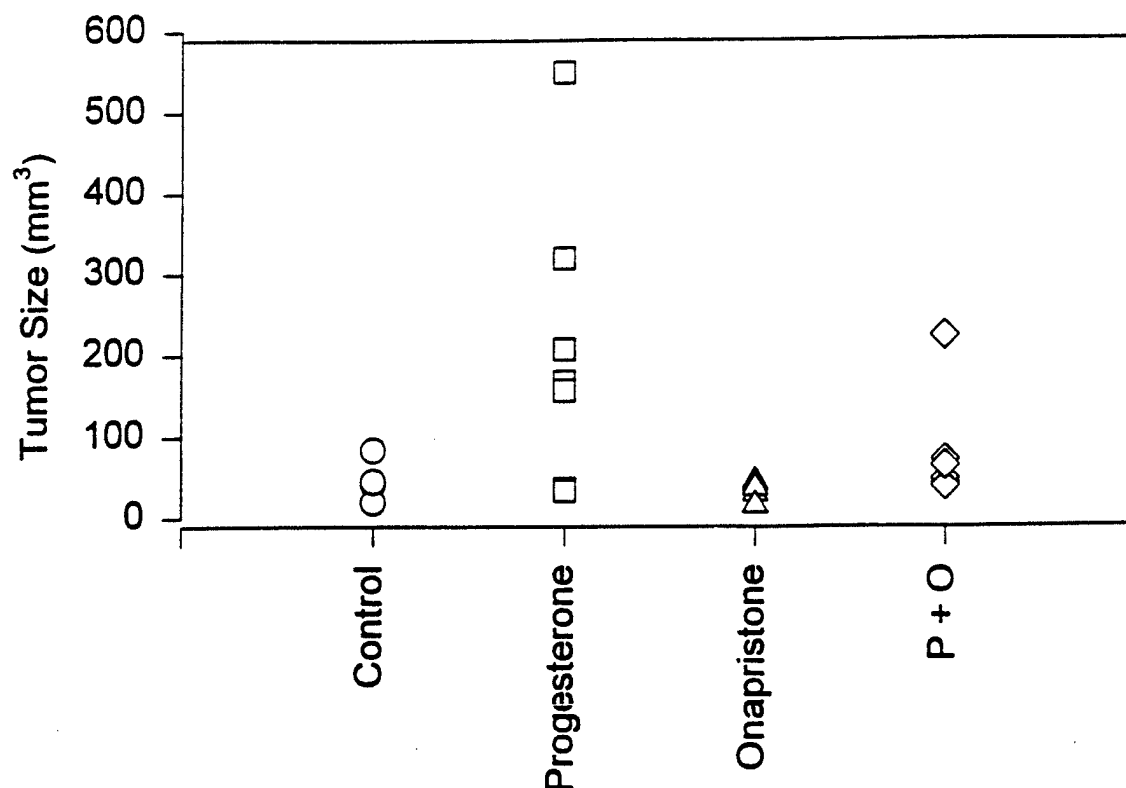


Effects of MPA and estradiol on TKS-7 tumor growth

	Tumor size (mm <sup>3</sup> + SE)	Tumor incidence (%)
Control	19 ± 8	50
MPA	47 ± 16	90
Estradiol	308 ± 90	100

5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. MPA, 1.5 mg/pellet/21 days; estradiol, 0.72 mg/pellet/60 days. The treatments with MPA were replaced every three weeks. Tumors were harvested after 6 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections. Statistical comparison for primary tumors was analyzed by Student's *t* test: MPA vs. control gave  $p < 0.05$ ; estradiol vs. control gave  $p < 0.01$ .

Fig. 3 . Effects of progesterone and onapristone on TKS-7 tumor growth

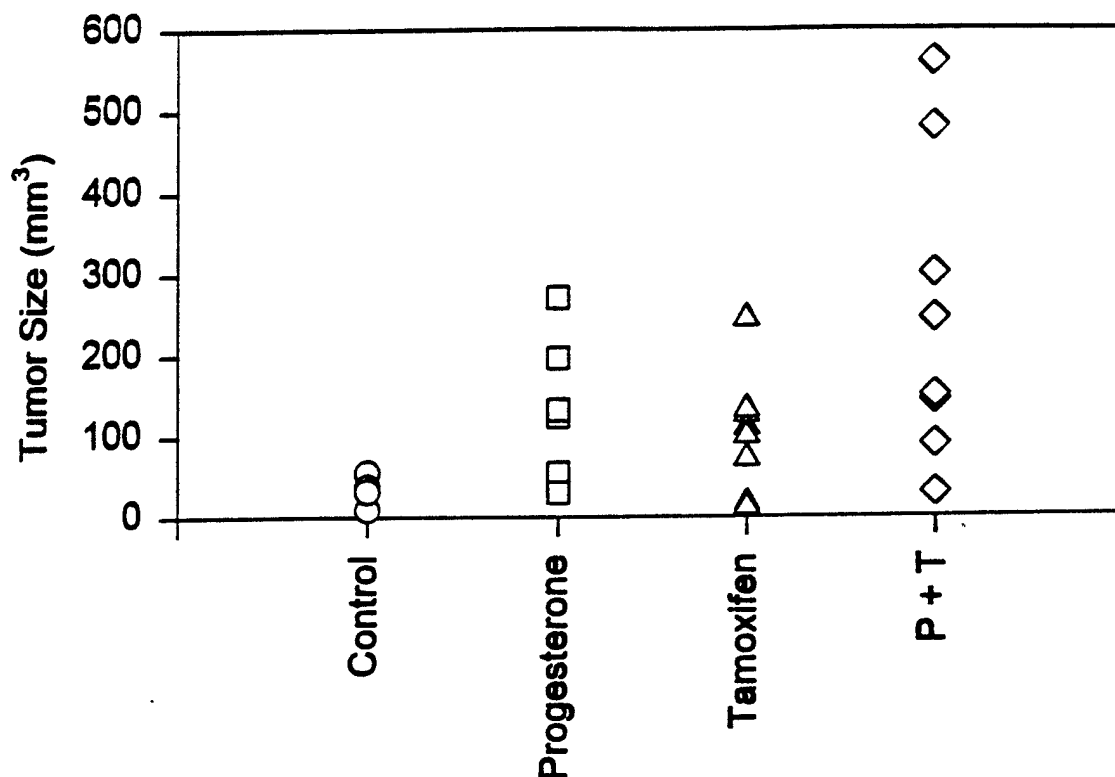


Effects of progesterone and onapristone on TKS-7 tumor growth

	Tumor size (mm <sup>3</sup> + SD)	Tumor incidence (%)	Lymph node metastasis
Control	42 ± 12	50	0
Progesterone (P)	211 ± 68	70	4
Onapristone (O)	33 ± 4	60	0
P+O	77 ± 25	70	1

5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. Progesterone, 10 mg/pellet/21 days; onapristone, 5 mg/pellet/21 days. The treatments with progesterone and onapristone were replaced every three weeks. Tumors were harvested after 8 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections. Statistical comparison for primary tumors was analyzed by Student's *t* test: progesterone vs. control gave  $p < 0.01$ ; P+O vs. progesterone gave  $p < 0.03$ . Lymph nodes near the cell injection sites were processed for histological analysis. Invasion of TKS-7 breast cancer cells to lymph node was clearly seen in four tumor injections in progesterone-treated mice. No lymph node metastasis were observed in either control or onapristone-treated mice; and only one lymph node metastasis was identified in progesterone- and onapristone-treated mice. The lymph node metastasis was defined by the appearance of the small island or foci of breast cancer cells which are bigger and lighter (hematoxylin stain) than the lymphatic cells (see Fig. 1C).

**Fig. 4. Effects of progesterone and tamoxifen on TKS-7 tumor growth**



Effects of progesterone and tamoxifen on TKS-7 tumor growth

	Tumor size (mm <sup>3</sup> + SD)	Tumor incidence (%)
Control	33 ± 17	40
Progesterone (P)	135 ± 36	70
Tamoxifen (T)	84 ± 24	100
P+T	259 ± 66	100

5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. Progesterone, 10 mg/pellet/21 days; tamoxifen, 5 mg/pellet/60 days. The treatments with progesterone were replaced every three weeks. Tumors were harvested after 6 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections. Statistical comparison for primary tumors was analyzed by Student's *t* test: progesterone vs. control gave  $p < 0.02$ ; Tamoxifen vs. control gave  $p < 0.05$ ; P+T vs. progesterone gave  $p < 0.05$ ; P+T vs. Tamoxifen gave  $p < 0.03$ .

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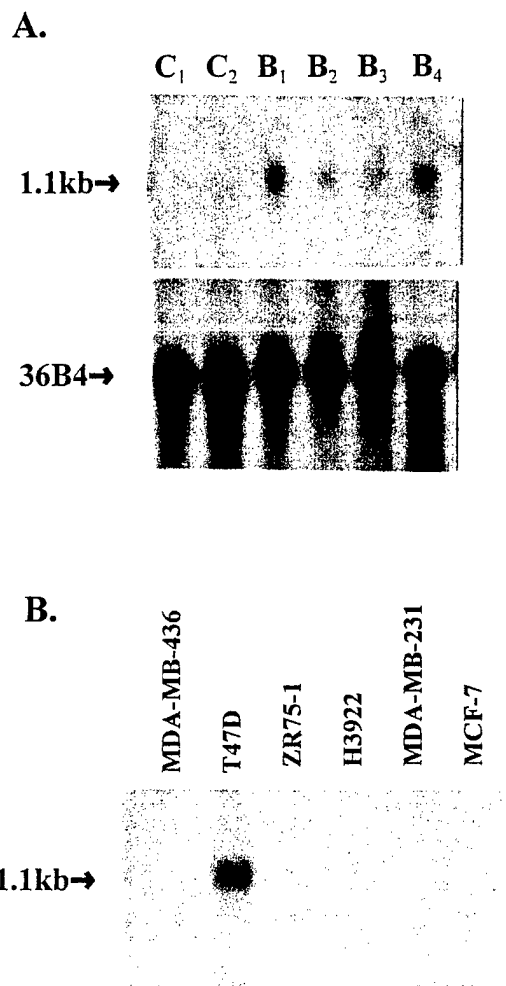
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      70              90              110
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      M K A L G V G F A T R Q V G N
      130              150              170
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V T K P T V I I S Q E G D K V V I R T L
      190              210              230
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S T F K N T E I S F Q L G E E F D E T T
      250              270              290
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A D D R N C K S V V S L D G D K L V H I
      310              330              350
CAGAAATGGGATGGCAAAGAAACAAATTTTGTAAAGAGAAATTAAGGATGGCAAATGGTT
Q K W D G K E T N F V R E I K D G K M V
      370              390              410
ATGACCCTTACTTTTGGTGATGTGGTTGCTGTTCCGCACTATGAGAAGGCATAAAAATGT
M T L T F G D V V A V R H Y E K A *
      430              450              470
CCCTGGTCGGGGCTTGGAAGAGCTCTTCAGTTTTTCTGTTTCCTCAAGTCTCAGTGCTAT
      490              510              530
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      610              630              650
TGTTTTATAATTTGAATTAAAGTTTGTCCCCCCCCCTTTTTTTTATAACAAGTGAAT
      670              690              710
ACATTTTATAATTTCTTTTGAATGTAAATCAAATTTGAATAAAAATCTTACACGTGAAA
      730
AAAAAAAAAAAA

```

**Fig. 6.** MRG cDNA sequence. The full-length cDNA was sequenced using ABI 373a Automated Fluorescent Sequencer method. The deduced amino acid sequence is shown under the DNA sequence. The terminal codon (TAA) is labelled with\*. Numbers refer to nucleic acid positions.

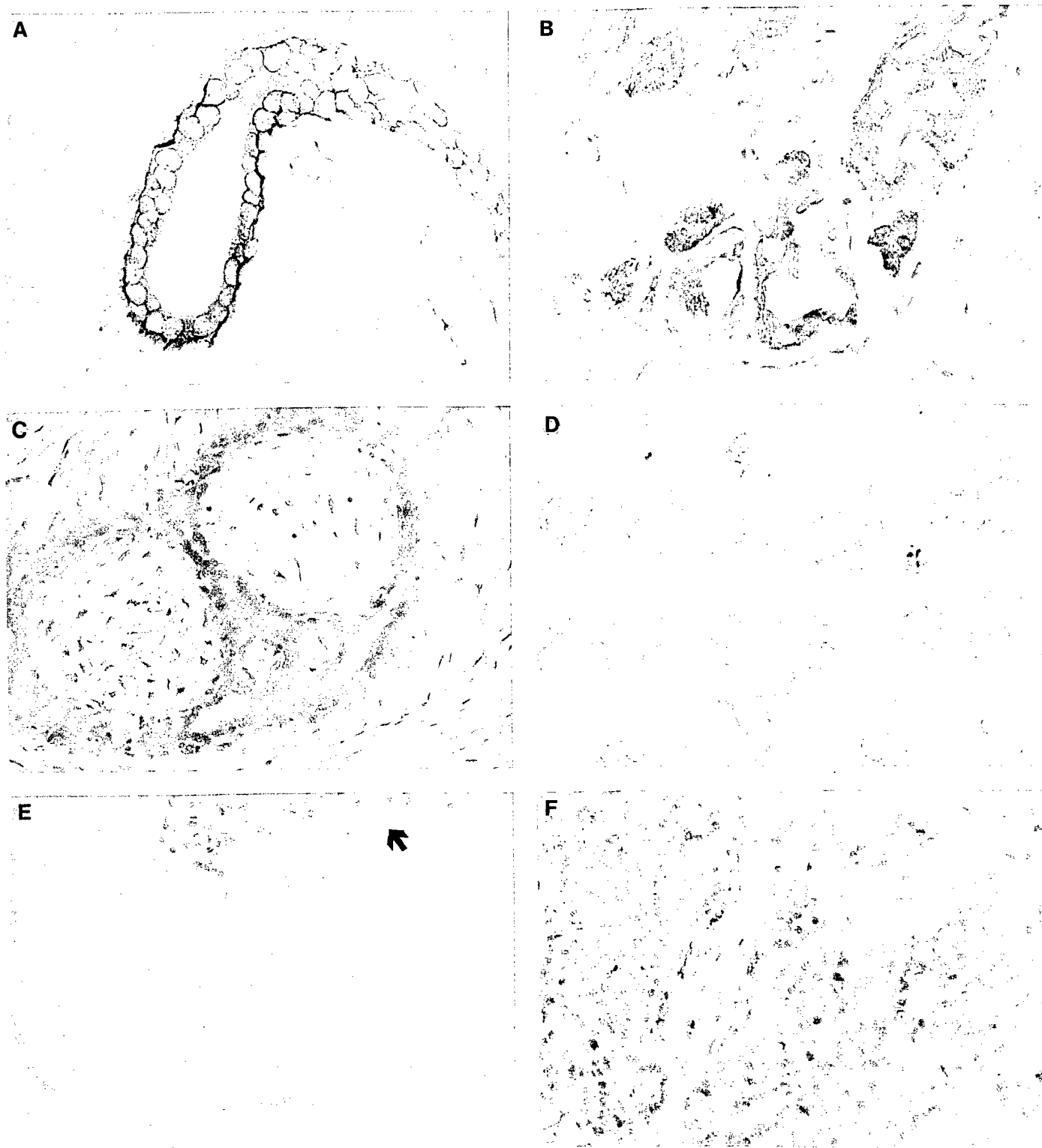
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1	MADAFVGTVK	LVDSKNFDDY	MKSLGVGFAT	RQVASMTKPT	Mouse MDGI
1	MVDAFLGTWK	LVDSKNFDDY	MKSLGVGFAT	RQVASMTKPT	H-FABP hu
1	MCDAFVGTVK	LVSENFDDY	MKEVGVGFAT	RKVAGMAKPN	A-FABP hu
41	VIISQEGDKV	VIRTLSTFKN	TEISFQLGEE	FDETTADDRN	MRG
41	TIIEKNGDTV	IIKTQSTFKN	TEISFKLGVE	FDETTADDRK	Bovine MDGI
41	TIIEVNGDTI	TIKTQSTFKN	TEINFQLGIE	FDEVTADDRK	Mouse MDGI
41	TIIEKNGDIL	TLKTHSTFKN	TEISFKLGVE	FDETTADDRK	H-FABP hu
41	MTISVNGDLV	TIRSESTFKN	TEISFKLGVE	FDEITADDRK	A-FABP hu
81	CKSVVSLDGD	KLVHIQKWDG	KETNFEVREIK	DGKMVMTLTF	MRG
81	VKSIIVTLDDG	KLVHVQKWNG	QETSLVREMV	DGKLILTLTH	Bovine MDGI
81	VKSLVTLDDG	KLIHVQKWNG	QETTTLREL	DGKLILTLTH	Mouse MDGI
81	VKSIIVTLDDG	KLVLHOKWDG	QETTLVRELI	DGKLILTLTH	H-FABP hu
81	VKSIITLDDG	ALVQVOKWDG	KSTTIKRKR	GDKLVVECV	A-FABP hu
121	GDVVAVRHYE	KA-			MRG
121	GTAVCTRTE	KQA			Bovine MDGI
121	GSVVSTRTE	KEA			Mouse MDGI
121	GTAVCTRTE	KEA			H-FABP hu
121	KGVTSTRVE	R-A			A-FABP hu

**Fig. 7.** Comparison of the predicted amino acid sequence of human MRG with mouse and bovine MDGI and human FABP. The amino acid sequence of MRG was aligned with those of bovine MDGI, mouse MDGI, human H-FABP, and human A-FABP. The available amino acid sequence of bovine and mouse MDGI and human H-FABP and A-FABP were obtained from the SwissProt data base and aligned with the MRG deduced sequence using the clustal method of the MegAlign Program from the DNASTAR software package. Conserved amino acids are shaded

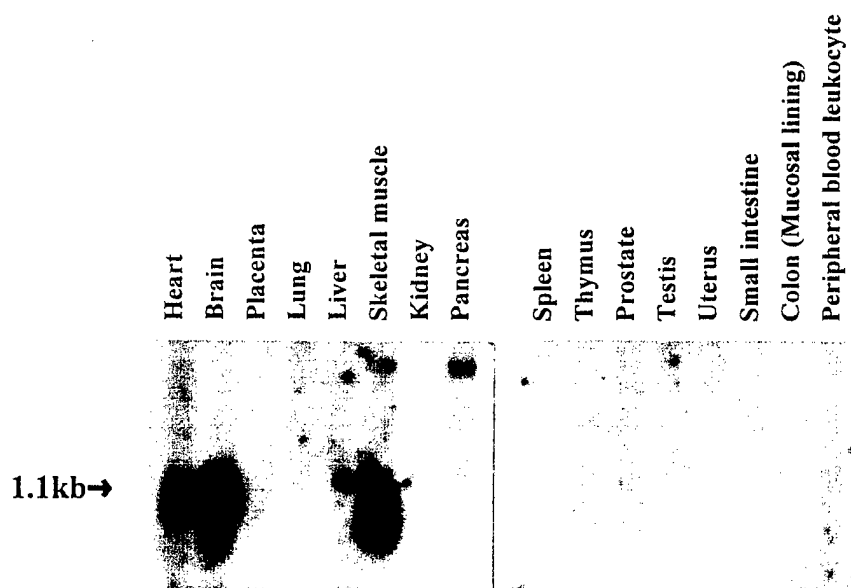


**Fig. 8.** (A) Northern blot analysis of MRG expression in human breast tissues. Total RNAs were prepared from two metastatic breast carcinomas (C represents carcinomas) and four normal breast reduction mammoplasty specimens (B represents normal breast). Each lane contained 30  $\mu$ g of total RNA. RNA integrity and loading control were ascertained by measuring house keeping gene 36B4. (B) MRG expression in human breast cancer cells. Only T47D cells showed a 1.1 kb MRG transcript. All breast cancer cell lines are from ATCC except H3922. H3922 was

Fig. 9



**Fig. 9.** *In situ* hybridization analysis of MRG expression in human breast. Cells labeled with *brown* indicate MRG gene expression. All Sections were counterstained lightly with hematoxylin for viewing negatively stained cells. (A) Normal ductal epithelial cells from a normal breast reduction mammoplasty specimen showed strong MRG expression. (B) A strong positive staining of MRG in normal lobular epithelial cells. (C) A benign fibroadenoma showed a strong MRG expression. (D) Benign hyperplasia showing a weak MRG staining; decreased MRG expression may be related to a proliferative stage. (E) Negative staining of a Comedo DCIS; arrow indicates the necrotic area. (F) Negative staining of an infiltrating breast cancer. Total 42 clinical breast specimens were analyzed; 19 out of 22 breast cancer samples were negative (-); 5 of 5 normal breast reduction mammoplasty samples were strong positive (++); 5 of 5 benign fibroadenomas were strong positive (++); and 6 of 10 benign hyperplasia were weakly positive (+) and the rest of 4 benign hyperplasia was negative (-). The normal breast section was also hybridized with the sense probe and no detectable background staining was observed at the same conditions for the anti-sense probe. All the sections presented in the figure derived from the same experiment.

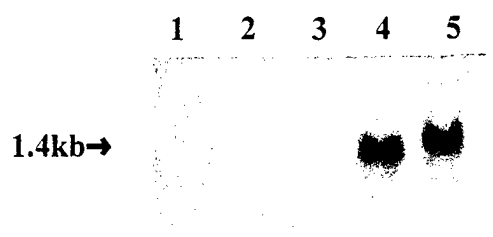


**Fig. 10.** The expression of MRG gene in a variety of normal human adult tissues. Two Northern blots containing approximately 2  $\mu$ g of poly (A) RNA per line from each of the above tissues were purchased from Clontech. Using a full-length cDNA hybridization probe, high abundance of 1.1 kb transcripts were detected in heart, brain, and skeletal muscle. A weak 2.2 kb transcripts were also detected in heart, skeletal muscle, and pancreas.

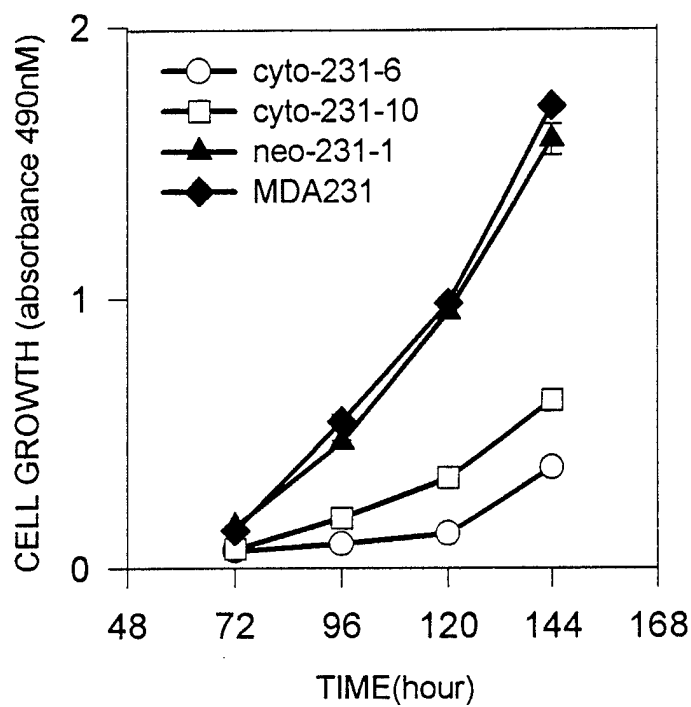


Fig. 11

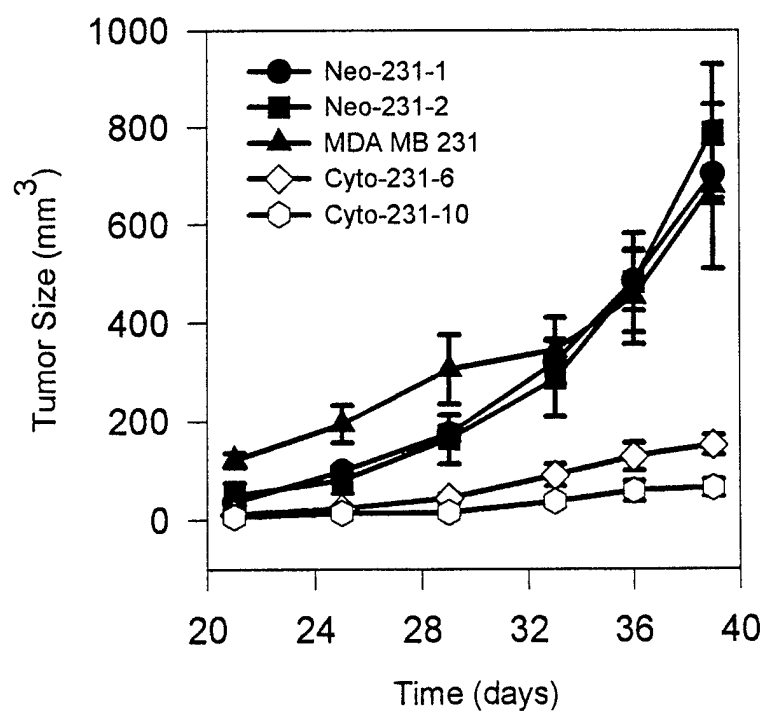
A.



B.



C.



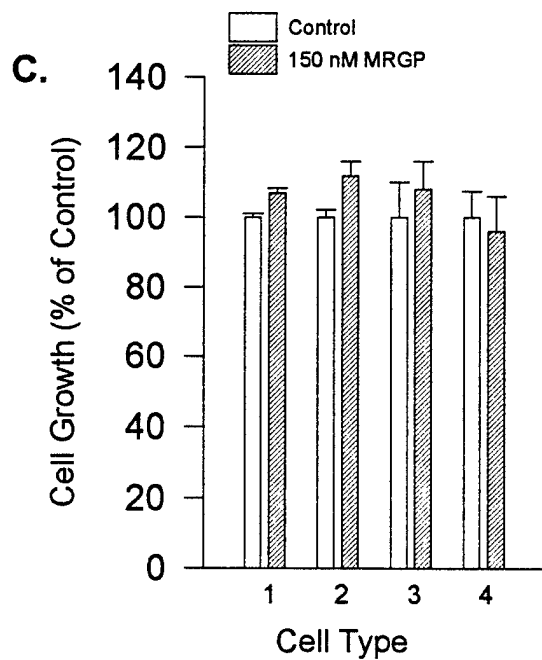
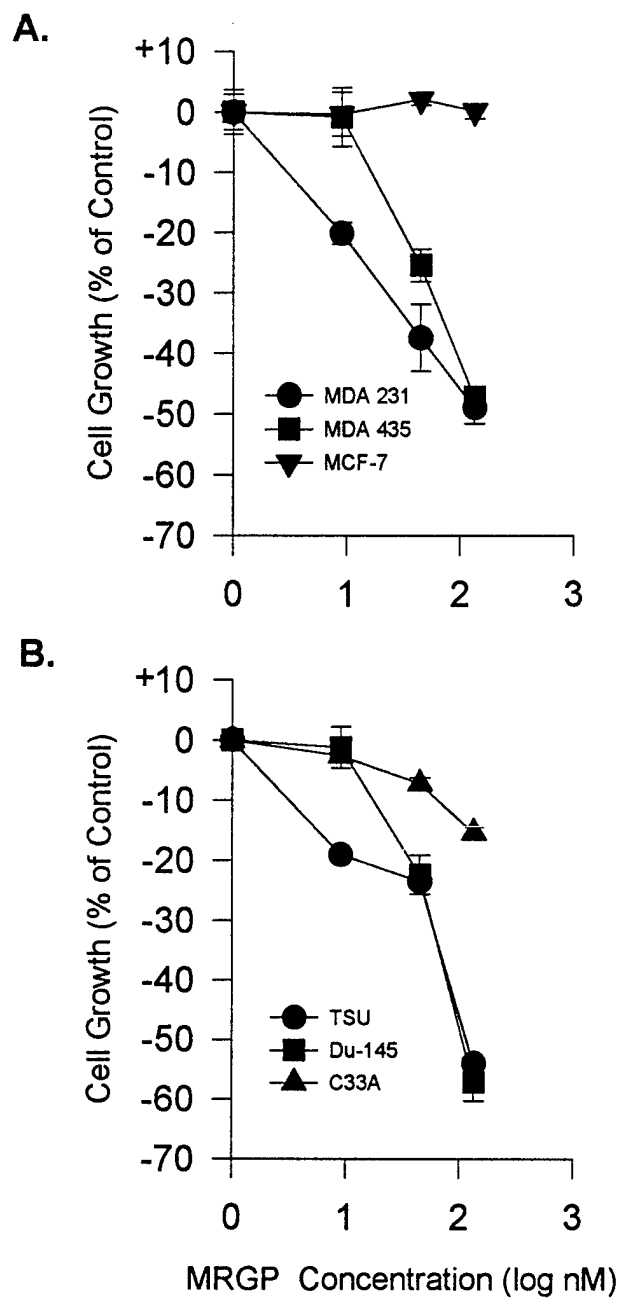
**Fig. 11.** Inhibition of breast cancer cell growth in vitro and tumor growth in nude mice by MRG expression.

(A) Northern blot analysis of MRG transfection of MDA-MB-231 cells. Total RNAs were isolated from parental MDA-MB-231 cells, two control pCI-neo transfected clones, and two MRG transfected clones. Strong MRG transcripts were detected in MRG positive clones. In contrast, no endogenous MRG transcripts were detected in all control clones and parental cells. Lane 1: parental MDA-MB-231 cells; lane 2: neo-231-1 clone; lane 3: neo-231-2 clone; lane 4: MRG-231-6 clone; lane 5: MRG-231-10 clone. The integrity of the RNAs and loading control were ascertained by visualization of the 18 S rRNA bands in stained gel (data not shown).

(B) Effect of MRG overexpression on growth. The growth rates of MRG positive clones were compared to that of MRG-negative clones in monolayer culture as described in "Materials and Methods." The number represents the mean  $\pm$  SE of three cultures.

(C) *In vivo* tumor growth of MDA-MB-231, neo-231-1, neo-231-2, MRG-231-6, and MRG-231 cells in the mammary fat pads of nude mice. Each point represents the mean  $\pm$  SE of tumors. Each of the five mice in each group received two injections, one on each side. The growth of developing tumors was measured at regular intervals for six weeks. Two independent experiments were done to confirm reproducibility and the results presented are representative of experiment 1 in Table 3.

Fig. 12



**Fig. 12.** Inhibition of cell growth by recombinant MRG protein (MRGP). Cells were treated with either different concentrations (A & B) or 150 nM (C) of MRGP for 6 days. Media were changed every two days. (A) Human breast cancer cells. (B) Human prostate and cervical cancer cells. TSU and Du-145, human prostate cancer cells. C33A, human cervical cancer cells. Cell growth was expressed as % of control: (+) stimulation; (-) inhibition. (C) 1: HMEC4144, normal human mammary epithelial cell. 2: HMVEC-d, normal human dermal microvascular endothelial cell. 3: WI-38, human lung fibroblast. 4: mouse Balb/c 3T3 fibroblast. Cell growth was expressed as % of control. All cell lines except HMEC4144 and HMVEC-d were cultured in the DMEM medium containing 5% FCS. HMEC4144 cells were cultured in mammary epithelial cell media from Clonetics. HMVEC-d cells were cultured in endothelial cell media from Clonetics.

**Method for Preparation of MRGP.** The full length MRG is amplified using standard PCR technique with primers corresponding to the 5' and 3' sequences of the gene (5' primer: gcg GGATCC CGTGGAGGCTTTCTGT; 3' primer: gcg GGTACC CCAGGGACATTTTAA). The amplified fragment was gel purified and digested with BamHI and Asp718. We have used the baculovirus expression vector, pA2-GP, derived from pVL94. The purified pA2-GP vector was ligated with amplified MRG coding sequence with T4 ligase. HB101 cells were transformed with the ligation mixture and positive clones were identified using PCR screening and restriction enzyme analysis. The DNA sequence was confirmed by automatic DNA sequencing of both strands. A recombinant virus was produced and purified. The purification of MRG was performed as follows: (1) Media supernatant, adjusted to pH 5.5, was first applied to tandem Poros HS/HQ columns (PerSeptive Biosystems) pre-equilibrated with 50 mM NaOAc, pH 5.5. (2) MRGP, collected in the flow through fraction, was adjusted to pH 8.0 and reapplied to the tandem Poros HS/HQ column pre-equilibrated with 20 mM Tris-HCl, pH 8.0. (3) MRGP, collected in the flow through fraction, was concentrated 50-fold using a Filtron 3000 MW cutoff tangential-flow system, and then separated on a Superdex-75 size exclusion column equilibrated with 10 mM NaOAc, pH 6.5. (4) Pooled MRGP fractions were applied to a Hydroxyapatite column equilibrated with 10 mM NaOAc, pH 6.5; the weak bound MRGP was eluted with 7.5 mM of  $K_2HPO_4$ , pH 6.8. (5) MRGP fractions were then separated on Superdex-75 size exclusion column equilibrated with 65 mM  $Na_2HPO_4$ , 100 mM NaCl, pH 7.2. MRGP fractions were pooled and found to be more than 98% pure by SDS-PAGE with endotoxin level less than 0.5 EU/mg. Purified MRGP was identified as three bands at 20, 22, and 25 kDa in the SDS-PAGE by silver staining. The sequences of all three bands were confirmed by N-terminal sequence analysis. The proteins were also analyzed for glycosylation by determining the monosaccharide content in a purified preparation and the N-linked sugar chains were confirmed. The different sizes of the three recombinant MRGP proteins are due to glycosylation.

# Antitumor activity of the novel human breast cancer growth inhibitor MRG

by

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Running title: MRG in human breast cancer

Abbreviations: EST, expressed sequence tag; MDGI, mammary derived growth inhibitor; MRG, MDGI-related gene; FABP, fatty acid binding protein; H-FABP, heart derived FABP; A-FABP, adipocyte derived FABP; DCIS, carcinoma *in situ*.

## Abstract

A novel human tumor growth inhibitor was identified by differential cDNA sequencing. This novel tumor suppressing factor has some homology to mouse mammary derived growth inhibitor (MDGI) and thus was named “MDGI-related gene” (MRG). MRG was found to be expressed in normal and benign human breast tissues but not in breast carcinomas. *In situ* hybridization analysis demonstrated stage-specific MRG expression as follows: MRG was undetectable in carcinomas, showed partial and weak expression in benign hyperplasia, but was expressed at high level in normal breast epithelial cells. To determine if MRG can modulate *in vivo* growth of human breast cancers, we transfected a full-length MRG cDNA into MDA-MB-231 human breast cancer cells and studied the orthotopic growth of MRG-transfected versus control clones in the mammary fat pad of athymic nude mice. Overexpression of MRG in human breast cancer cells significantly suppressed tumor cell growth *in vitro* and in an orthotopic nude mouse model. These results suggest that MRG has a tumor suppressing activity and its loss may thus be involved in the development and progression of breast cancer.

## Introduction

Development of cancer and subsequent malignant progression are associated with multiple genetic changes that result in qualitative and quantitative alterations in individual gene expression (1). Many of these quantitative genetic changes manifest them as alterations in the cellular complement of novel transcribed mRNAs. Identification of these mRNAs, if sufficiently characterized, could provide clinically useful information for patient management and prognosis while enhancing our understanding of cancer pathogenesis. The outcome of cancer growth can be determined by the balance between growth factors or oncogenes and growth inhibitors or tumor suppressor genes in the tumor environment. The list of growth inhibitors or tumor suppressor genes remains slim in comparison to the hundred-odd growth factors or oncogenes currently recognized.

Breast cancer evolves by clonal selection of cells that acquire multiple genetic changes. One proposed model for such accumulation of multiple genetic changes suggests that breast cancer, like colon cancer (2), develops through defined morphologically distinguishable stages beginning with benign hyperplasia, progresses to atypical hyperplasia, which leads to carcinoma *in situ*, and finally to an invasive carcinoma (3). It has been proposed that the loss of tumor suppressors or growth inhibitors that locally signal growth cessation and stimulate differentiation of the developing mammary epithelium plays a critical role in this sequential development of breast cancer (4-5). Currently, only few naturally occurring growth inhibitors have been identified. These include TGF- $\beta$  (6-7), interferons (8), tumor necrosis factor (TNF) (9), and MDGI (10-13) for mammary epithelial cells. Their roles in the onset and progression of both hereditary and sporadic breast cancer are not defined.

We have used differential cDNA sequencing approach (14) to search for potential tumor suppressor genes whose expressions are decreased during breast cancer onset and progression.

Differential cDNA sequencing is an expressed sequence tag (EST) sequencing-based method of differential expression cloning. Since the introduction of the EST sequencing approach, many novel human genes have been discovered (15,23). The advantage of this methodology, compared to isolation and sequencing of individual cDNAs, is that a large number of sequences can be "cataloged" with small amounts of sequencing data. Therefore, unlike differential display and subtractive hybridization which require the relative time- and labor-intensive steps of subcloning, library screening, and cDNA sequencing of individual genes (16-17), creation of EST libraries is a rapid method used to identify or "tag" sequences that are expressed in specific tissues (15,18). A novel growth inhibitor MRG with sequence homology to MDGI for breast cancer was identified as being down-regulated in breast cancer as compared with normal breast.

MDGI is a mammary epithelial cell growth inhibitor and differentiation factor initially identified and purified from Ehrlich ascites mammary carcinoma cells (10), and then from the lactating bovine mammary gland (11-12) and from cows milk (13). MDGI revealed no homology to any other known growth inhibitors (19); rather, MDGI belongs to a multigene family of fatty acid binding protein (FABP) (20). So far, only bovine (11-12) and mouse (21-22) MDGI was identified and characterized. In this study, the expression of MRG in human breast and its antitumor activity were characterized. Our results suggest that MRG is a tumor suppressor gene and its loss of expression in breast carcinoma may contribute to breast cancer onset and progression.



## Materials and Methods

**Reagents.** Restriction enzymes, T7 polymerase, random primer DNA labeling kit, and digoxigenin-labeled nucleotides were obtained from Boehringer Mannheim, Indianapolis.  $^{32}\text{P}$ -dATP was purchased from Amersham.

**Molecular cloning of MRG full-length cDNA sequence.** We have used EST-based automatic differential cDNA sequence analysis as we previously described (14) to search for new genes differentially expressed in breast cancer versus normal breast. Briefly, a data base containing approximately one million human partial cDNA sequences (ESTs) has been established in a collaborative effort between the Institute for Genomic Research and Human Genome Science Inc., using high throughput automated DNA sequence analysis of randomly selected human cDNA clones (23). RNAs from a breast carcinoma and patient-matched normal breast were isolated and subjected to preparation of cDNA libraries. EST automated DNA sequence analysis was performed on randomly selected cDNA clones. The ESTs with overlapping sequences were grouped into unique EST groups. Each EST group may represent a gene or a family of sequence-related genes. Each unique EST group was analyzed for its relative expression by examining the number of expressed individual EST in the libraries of normal vs. cancerous breast. The numbers of EST hits in the libraries reflect the relative expression or mRNA transcript copy numbers of the EST. There were more than 5,000 EST groups that were analyzed for quantitative comparison of EST hits in the pair of cDNA libraries from normal breast versus breast cancer by examining the expression of individual EST sequences. Among several differential expressed EST groups (14), one EST with full-length cDNA sequence from human normal breast library, which demonstrated homology to MDGI, was completely sequenced and named as MRG.

***In situ* hybridization.** *In situ* hybridization was carried out as we previously described (14).

Briefly, deparaffinized and acid-treated human breast sections (5- $\mu$ m thick) were treated with proteinase K, pre-hybridized, and hybridized overnight with digoxigenin labeled antisense transcripts from a MRG cDNA insert. The MRG antisense probe is a 731 bp full-length fragment. The probe was generated by EcoR1 digestion of MRG cDNA plasmid and followed by T7 polymerase. Hybridization was followed by RNase treatment and three stringent washes. Sections were incubated with mouse anti-digoxigenin antibodies (Boehringer Mannheim) followed by the incubation with biotin-conjugated secondary rabbit anti-mouse antibodies (DAKO). The colorimetric detection was performed by using a standard indirect streptavidin-biotin immunoreaction method by DAKO's Universal LSAB Kit according to manufacturers' instructions. There were some variations in staining intensity for MRG expression among the specimens. Since the colorimetric *in situ* hybridization is not quantitative, the tissue samples were classified into three classes: negative (-), strong positive (++), and weakly positive (+). The negative cases were confirmed with at least two independent experiments. All stainings were reviewed by two pathologists.

**Transfection.** The full-length MRG cDNA was inserted into a pCI-neo mammalian expression vector; and the resulting vector was transfected into MDA-MB-231 cells as we described previously (24). Subsequent to transfection, G418 selection, and cloning by limiting dilution, several subclones of MDA-MB-231 cells were obtained. These G418-resistant clones were expanded into individual cell lines and used as a source for RNA analysis. Clones were initially screened by *in situ* hybridization with a specific MRG antisense probe, and the positive clones were subjected to Northern blot analysis.

**Northern blot analysis.** Detection of MRG mRNA expression was analyzed by Northern blot as we described previously (24).

**In vitro assay for cell growth.** Exponentially growing cultures of different MDA-MB-231

clones were detached with trypsin, and the trypsin was neutralized with DMEM-10% serum. Cells were counted, diluted, and seeded in triplicate at 3,000 cells per well (24-well plate) in 1 ml DMEM-5% serum. Cell growth was measured using CellTiter 96™ Aqueous Non-Radioactive cell proliferation Assay Kit (Promega).

**Tumor growth in athymic nude mice.** Nude mouse tumorigenic assay was performed as we previously described (25). Briefly, cells were grown to 80-90% of confluence in 150 cm<sup>2</sup> dishes and were harvested by incubation with 5 mM EDTA in PBS. The EDTA was neutralized with medium containing serum. The cells were washed twice with serum-free medium, counted, and re-suspended in serum-free DMEM at a concentration of  $2.7 \times 10^6$  cells/ml. Approximately  $0.4 \times 10^6$  cells (0.15 ml) were injected into 5-6 week old female athymic nude mice (Frederick Cancer Research and Development Center, Frederick, MD). Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. The animals were ear tagged. Primary tumor growth was assessed by measuring the volume of each tumor at weekly intervals. Tumor size was determined at intervals by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor cell clone at each time point. Animals were sacrificed between 32-40 days after injection, when the largest tumors reached about 15 mm in diameter.

**Statistical Analysis.** Values were expressed as means  $\pm$  standard errors (SEs). Where appropriate, comparisons were made using the two-tailed Student's *t-test*.

## Results

**Molecular cloning of MRG cDNA.** We generated cDNA libraries from breast cancer biopsy specimen and normal breast and analyzed these libraries by EST-based differential cDNA sequencing. ESTs were sequence analyzed and grouped to different groups based on sequence overlapping, and each unique EST group was first analyzed for relative expression in the cDNA libraries from normal breast versus breast cancer and then subjected to tissue-specific expression by examining tissue origins of individual EST sequences against approximately one million of ESTs derived from a variety of different tissue types. As previously demonstrated (14), we identified three classes of EST groups that were differentially expressed in normal breast versus breast cancer: (1) genes more abundant in breast cancer than in normal breast; (2) genes that are more abundant in normal breast than in breast cancer; and (3) genes that are selectively expressed in breast relative to other tissue types. Within the second class, the automated screening revealed a group of 25 ESTs encoding a novel gene with homology to mouse MDGI (greater than 30% homology). Of the 25 distinctive MDGI-related EST clones, 4 ESTs were derived from normal breast library, and the rest of ESTs were derived from fetal brain library. No ESTs were derived from breast cancer library. After sequencing of these cDNA fragments, one clone containing a start codon (ATG) and an open reading frame was isolated and characterized.

The nucleotide sequence determined from this clone and the predicted corresponding amino acid sequences are shown in Fig. 1. The full-length cDNA sequence contains 731 bp with 396 bp open reading frame; 75 bp in the 5' untranslated region; and 260 bp of 3' untranslated sequence. The open reading frame extends from the initiation A<sub>76</sub>TG codon to TAA<sub>474</sub> stop codon. The open reading frame encodes a protein of 132 amino acids. The deduced amino acid sequence predicts a protein with an isoelectric point of 5.4. Comparisons of the predicted amino acid sequence with the

sequence of similar human proteins are shown in Fig. 2. After optimal alignment, the putative protein shows 63.4% amino acid identity to mouse MDGI, 68.9% identity to bovine MDGI, 66.2% identity to heart derived fatty acid binding protein (H-FABP), and 56.8% identity to adipocyte derived fatty acid binding protein (A-FABP). The extensive similarity of the predicted amino acid sequence with MDGI suggests that the putative new protein is a MDGI-related and therefore this gene is designated as MRG.

**Expression of MRG in human breast epithelial cells.** Since the differential cDNA sequencing revealed MRG expression in normal breast library but not in breast cancer library and its sequence homology to MDGI, a previously described bovine- and mouse-derived growth inhibitor for mammary epithelial cells, we speculate that MRG is a potential tumor suppressor gene or growth inhibitor for mammary gland. In an attempt to evaluate the potential biological significance of MRG on human breast cancer development and progression, we first studied MRG gene expression in human biopsy samples from breast carcinoma and normal breast tissue. Northern blot analysis (Fig. 3A) allows the detection of MRG expression in all four normal breast reduction mammoplasty specimens, but not in two malignant breast cancer samples. The presence of MRG in normal breast and the loss of MRG expression in breast carcinomas suggest a possible role of down-regulation of MRG in the development of breast cancer. The expression of MRG was also investigated in some human breast cancer cell lines (Fig. 3B). No expression of MRG was detected in any of the tested human breast cancer cell lines except T47D cells.

In order to localize the cellular source of MRG expression and to further assess the biological relevance of the loss of MRG expression in breast cancers, we next performed *in situ* hybridization on fixed sections from a variety of different human breast specimens. In these experiments, we examined two aspects of MRG expression, including the tissue localization (stromal versus epithelial)

and the correlation of the loss of MRG expression and breast cancer malignant phenotype. Fig. 4 shows a representative *in situ* hybridization for MRG. We found a strongly positive MRG hybridization in epithelial cells of normal mammary gland (Fig. 4A & B) and benign fibroadenoma (Fig. 4C). The expression of MRG mRNA was detectable in the epithelial cells in all 5 reduction mammoplasty specimens and in 5 benign fibroadenomas. In contrast, expression of MRG was absent in 9 out of 10 cases of infiltrating breast carcinomas and in 10 out of 12 ductal carcinoma *in situ* (DCIS) samples. A representative negative staining of MRG in DCIS neoplastic epithelial cells (Fig. 4E) and in an infiltrating breast carcinoma (Fig. 4F) are presented. These *in situ* hybridization results are consistent with the Northern blot analysis which showed MRG expression in normal breast but not in breast carcinomas.

It is interesting to note that although a strong MRG signal was easily detected in the breast epithelial cells of normal gland and benign fibroadenomas, the benign breast hyperplasia showed different MRG expression patterns. Among 10 benign hyperplasias, 4 specimens were negative and 6 specimens were lightly stained for MRG expression. As illustrated in Fig. 4D, the intensity of MRG staining in a benign hyperplasia was greatly reduced compared to normal mammary gland (Fig. 4A & B) or benign fibroadenoma (Fig. 4C). The decreased MRG expressions in hyperplasia and the loss of MRG expression in breast carcinoma suggest that expression of MRG is stage-specific in breast: down-regulated during the proliferative stage and lost in breast cancer.

**Tissue expression.** Tissue-specific transcription of MRG was examined by Northern blot on 2 µg of poly (A) RNAs from various human adult tissues (Fig. 5). As expected, the Northern blot showed that MRG was abundantly expressed as a 1.1- kb transcript in brain, in which 21 MDGI-related ESTs were identified, and in heart and skeletal muscle which are the rich source for H-FABP. A 2.2 kb transcript with much lower accumulations in their relative intensity was also detected

in heart, skeletal muscle, and pancreas. By contrast, none of them was present in other specimens analyzed, such as placenta, lung, liver, kidney, spleen, thymus, prostate, testis, uterus, colon, and small intestine.

**Transfection and selection of MRG positive clones.** Since we demonstrated a loss of MRG expression in breast cancer compared with normal or benign breast, we next asked if we could suppress breast cancer growth by overexpression of MRG gene in breast cancer cells. We selected MDA-MB-231 cell line as recipient for MRG mediated gene transfection because of 1) its lack of detectable MRG transcript; and 2) more aggressive and highly tumorigenic behavior in nude mice. Cells were transfected with a plasmid vector containing a neomycin resistance gene (neo-231 clones), or the same vector containing a full-length MRG cDNA (MRG-231 clones). Clones were initially screened by *in situ* hybridization on slides with a specific MRG antisense probe (data not shown), and the positive clones were subjected to Northern blot analysis. Six MRG-231 clones were picked up by *in situ* hybridization. Fig 6. shows the Northern blot analysis of MRG expression in selected clones. All two selected MRG-231 clones MRG-231-6 and MRG-231-10 expressed MRG mRNA transcripts. In contrast, none of the parental MDA-MB-231 cells or neo-231-1 and neo-231-2 clones produced any detectable MRG transcripts.

**Effect of MRG transfection on cell growth.** The growth curve of MRG-231 cells was compared to that of MRG negative MDA-MB-231 and neo-231-1 cells in a monolayer culture (Fig. 7). Cell growth was inhibited approximately 65-75% ( $p < 0.001$  by Student's *t-test*) in the MRG-231-6 and MRG-231-10 cells compared to the parental MDA-MB-231 and neo-231-1 cells. There was no significant difference in the growth pattern between parental MDA-MB-231 cells and neo-231-1 cells.

**Suppression of tumorigenicity by MRG transfection.** To study the effect of MRG

expression on tumorigenicity, The tumorigenicities of MRG-231 clones were determined in comparison with parental MDA-MB-231 cells and neo-231 clones in an orthotopic nude mouse model. Two independent experiments were done to confirm reproducibility and the data were shown in Table 1. After a lag phase of approximately 10 days, mice given implants of both MRG positive and MRG negative cells developed tumors. There was no significant difference in tumor incidence among the groups. Starting at about 30 days after inoculation, great level of tumor necrosis was observed in tumors derived from MDA-MB-231, neo-231-1, and neo-231-2 cells. The same breast cancer cells transfected with MRG, however, were significantly inhibited in their tumor growth; and either no or low level of tumor necrosis was observed. The mean volume of MRG-231-6 and MRG-231-10 tumors was only 21% of that in parental MDA-MB-231 tumors, 24% of that in neo-231-1 tumors, and 18% of that in neo-231-2 tumors, respectively ( $P < 0.005$  by Student's *t* test). Fig. 8 shows growth kinetics of one representative experiment from parental MDA-MB-231, neo-231-1, neo-231-2, MRG-231-6, and MRG-231-10 tumors. After a slow growth phase of 25 days, tumors from both parental MDA-MB-231 cells and two neo-MDA-231 clones increased in volume at an exponential rate. In contrast, the growths of MRG-231-6 and MRG-231-10 cells were dramatically inhibited. Thus, the tumorigenicity of the human breast cancer cells was significantly inhibited by expression of MRG.



## Discussion

Many breast tumors go through a series of events from the time of initial detection to the formation of the lethal metastatic stage. Peptides that locally signal growth cessation and stimulate differentiation of the developing epithelium is very important for mammary gland development and preventing cancer formation. Identification of these peptides, if sufficiently characterized, may yield novel molecular markers which may be useful in the diagnosis and treatment of breast cancer. The differential cDNA sequencing method (14) was applied to the isolation of transcriptionally regulated genes involved in the development of human breast cancer progression. The gene encodes MRG, a MDGI related growth inhibitor for breast epithelial cells, expressed in normal or benign breast epithelial cells but absent in malignant breast epithelial cells. Overexpression of MRG in MDA-MB-231 human breast cancer cells inhibited cell growth. When injected orthotopically into nude mice, MRG transfectants were significantly inhibited in tumor growth as compared with controls.

Using *in situ* hybridization analysis, we have demonstrated the expression of MRG transcripts in epithelial cells of normal and benign breast but no in the neoplastic epithelial cells of breast carcinoma *in situ* and infiltrating breast carcinoma. The high expression (10 of 10) of MRG gene in epithelial cells of normal breast and benign fibroadenoma compared to extremely low expression (3/22) in the malignant breast epithelial suggests that the loss or down-regulation of MRG expression is associated with breast cancer development. This implication is further supported by the detection of partial and weak MRG expression in benign hyperplasia. Only 6 out of 10 benign hyperplasias were stained for MRG expression. In addition, the decreased MRG is evident in these rapidly proliferating nonmalignant breast lesions (Fig. 4D). It is likely that the down-regulation of MRG expression is the consequence of cellular proliferation.

It will be interesting to investigate if the down regulation of MRG in a benign hyperplasia

may indicate a malignant progression to an atypical hyperplasia and subsequent development of a breast carcinoma. If MRG expression can provide some prognostic information on distinguishing a benign hyperplasia which is not likely to have malignant progression and an atypical hyperplasia which is likely to progress to a carcinoma, this will help to direct the treatment strategies and to reduce the development of breast cancer.

MRG revealed no sequence homology to any of the hitherto known growth inhibitors except partial homology to mouse and bovine MDGI. Until now, only bovine (11-12) and mouse MDGI (21-22) have been identified and characterized. Studies of mouse and bovine MDGI suggest several potential functions of MDGI on growth and differentiation of mammary gland. Recombinant and native mouse MDGI specifically inhibit the growth of normal mouse mammary epithelial cells (MEC), and promote morphological differentiation and milk protein synthesis (26). Selective inhibition of endogenous MDGI expression in mouse MEC by use of antisense oligonucleotides suppresses alveolar budding and impairs  $\beta$ -casein synthesis in organ cultures (26). Increasing amounts of MDGI mRNA were detected in the epithelium of developing lobules and in terminal parts of ducts and lobuloalveolar epithelial cells of differentiated glands (27). These data suggest a local role of MDGI as a growth inhibitor in mediating or complementing hormonal action during differentiation (28-29). In this regard, MDGI is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells (30), which may be involved in the protective effect of early parity on a subsequent breast cancer incidence. Previously, bovine MDGI has been demonstrated to inhibit breast cancer growth (31). Within the same content, we demonstrated that overexpression of MRG in MDA-MB-231 human breast cancer cells significantly inhibited their tumor growth by 4-6-fold as compared to MRG negative control tumors in nude mice. This MRG-mediated *in vivo* tumor growth inhibition is consistent to its *in vitro* inhibition of cell growth.

MDGI revealed extensive sequence homology to FABP (20). The literature abounds with conflicting data about identity of MDGI and FABP in the mammary gland. A striking homology was evident between bovine MDGI and H-FABP, which differ only in seven positions of the amino acid sequence (32). In fact, the previously described sequence for MDGI (26) is the mix of H-FABP and A-FABP proteins (33). Furthermore, mouse MDGI and mouse FABP-muscle and -heart are also assumed to be the same gene (34) based on the complete cDNA and protein homologies. These results suggest that the functions of previously described MDGI are exerted by FABP (33). Unlike MDGI which has almost identical sequence to H-FABP, MRG has relatively low sequence identity to human H-FABP (66%). In addition, in contrast to previously described MDGI, the growth inhibitory activity of partially purified recombinant MRG protein from baculovirus expression system is not limited to mammary cells, because the growth of human prostate cancer and cervical cancer cells were also inhibited by MRG protein.<sup>1</sup> These results suggest that MRG may represent a MDGI-related but different class of growth inhibitor. Thus, along with interferons, TGF- $\beta$ , TNF, and MDGI, MRG is one of the few naturally occurring growth inhibitors for mammary epithelium.

The magnitude of the tumor suppressing activity of MRG is comparable to that previously observed for *Rb* and *p53* (35). The loss of MRG expression in malignant breast cancers and the inhibition of breast tumor growth by re-expression of MRG suggests that MRG is one of the growth inhibitors that locally signal growth cessation of the mammary gland. In fact, the human homologue of bovine MDGI has been mapped to chromosome 1p32-35 (31), a locus previously shown to exhibit frequent loss of heterozygosity in human sporadic breast cancer (36-37). Therefore, the loss or down-regulation of MRG or its receptor may lead to abnormal growth and the development of breast cancer.

<sup>1</sup> G. Xiao and Y. E. Shi, unpublished data.

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## Figure legend

Fig. 1. MRG cDNA sequence. The full-length cDNA was sequenced using ABI 373a Automated Fluorescent Sequencer method. The deduced amino acid sequence is shown under the DNA sequence. The terminal codon (TAA) is labelled with\*. Numbers refer to nucleic acid positions.

Fig. 2. Comparison of the predicted amino acid sequence of human MRG with mouse and bovine MDGI and human FABP. The amino acid sequence of MRG was aligned with those of bovine MDGI, mouse MDGI, human H-FABP, and human A-FABP. The available amino acid sequence of bovine and mouse MDGI and human H-FABP and A-FABP were obtained from the SwissProt data base and aligned with the MRG deduced sequence using the clustal method of the MegAlign Program from the DNASTAR software package. Conserved amino acids are shaded.

Fig. 3. (A) Northern blot analysis of MRG expression in human breast tissues. Total RNAs were prepared from two metastatic breast carcinomas (C represents carcinomas) and four normal breast reduction mammoplasty specimens (B represents normal breast). Each lane contained 30 µg of total RNA. RNA integrity and loading control were ascertained by measuring house keeping gene 36B4. (B) MRG expression in human breast cancer cells. Only T47D cells showed a 1.1 kb MRG transcript. All breast cancer cell lines are from ATCC except H3922. H3922 was isolated from an infiltrating ductal carcinoma at Bristol-Myers Squibb Pharmaceutical Research Institute.

Fig. 4. *In situ* hybridization analysis of MRG expression in human breast. Cells labeled with *brown* indicate MRG gene expression. All Sections were counterstained lightly with hematoxylin for viewing

negatively stained cells. (A) Normal ductal epithelial cells from a normal breast reduction mammoplasty specimen showed strong MRG expression. (B) A strong positive staining of MRG in normal lobular epithelial cells. (C) A benign fibroadenoma showed a strong MRG expression. (D) Benign hyperplasia showing a weak MRG staining; decreased MRG expression may be related to a proliferative stage. (E) Negative staining of a Comedo DCIS; arrow indicates the necrotic area. (F) Negative staining of an infiltrating breast cancer. Total 42 clinical breast specimens were analyzed; 19 out of 22 breast cancer samples were negative (-); 5 of 5 normal breast reduction mammoplasty samples were strong positive (++); 5 of 5 benign fibroadenomas were strong positive (++); and 6 of 10 benign hyperplasia were weakly positive (+) and the rest of 4 benign hyperplasia was negative (-). The normal breast section was also hybridized with the sense probe and no detectable background staining was observed at the same conditions for the anti-sense probe. All the sections presented in the figure derived from the same experiment.

Fig. 5. The expression of MRG gene in a variety of normal human adult tissues. Two Northern blots containing approximately 2  $\mu$ g of poly (A) RNA per line from each of the above tissues were purchased from Clontech. Using a full-length cDNA hybridization probe, high abundance of 1.1 kb transcripts were detected in heart, brain, and skeletal muscle. A weak 2.2 kb transcripts were also detected in heart, skeletal muscle, and pancreas.

Fig. 6. Northern blot analysis of MRG transfection of MDA-MB-231 cells. Total RNAs were isolated from parental MDA-MB-231 cells, two control pCI-neo transfected clones, and two MRG transfected clones. Strong MRG transcripts were detected in MRG positive clones. In contrast, no endogenous

MRG transcripts were detected in all control clones and parental cells. Lane 1: parental MDA-MB-231 cells; lane 2: neo-231-1 clone; lane 3: neo-231-2 clone; lane 4: MRG-231-6 clone; lane 5: MRG-231-10 clone. The integrity of the RNAs and loading control were ascertained by visualization of the 18 S rRNA bands in stained gel (data not shown).

Fig. 7. Effect of MRG overexpression on growth. The growth rates of MRG positive clones were compared to that of MRG-negative clones in monolayer culture as described in "Materials and Methods." The number represents the mean  $\pm$  SE of three cultures.

Fig. 8. *In vivo* tumor growth of MDA-MB-231, neo-231-1, neo-231-2, MRG-231-6, and MRG-231 cells in the mammary fat pads of nude mice. Each point represents the mean  $\pm$  SE of tumors. Each of the five mice in each group received two injections, one on each side. The growth of developing tumors was measured at regular intervals for six weeks. Two independent experiments were done to confirm reproducibility and the results presented are representative of experiment 1 in Table 1.

**Table 1.** Effects of MRG expression on tumor incidences and sizes of MDA-MB-231 cells

Experiment	Treatment Group	Tumor Incidence Tumor/Total (%)	Tumor Vol (mm <sup>3</sup> ) of Primary Size
1	MDA-MB-231	9/10 (90)	679 ± 169
	neo-231-1	8/10 (80)	704 ± 62
	neo-231-2	8/10 (80)	792 ± 59
	MRG-231-6	9/10 (90)	151 ± 29
	MRG-231-10	8/10 (80)	64 ± 17
2	MDA-MB-231	10/10 (100)	824 ± 215
	neo-231-1	9/10 (90)	645 ± 157
	neo-231-2	9/10 (90)	957 ± 182
	MRG-231-6	8/10 (80)	212 ± 41
	MRG-231-10	9/10 (90)	179 ± 53

Four hundred thousand of the cells were injected at day one into the mammary fat pads, and tumor volumes were determined as described in Materials and Methods. Volumes are expressed as means ± SEs (number of tumors assayed). For both experiments, total 10 injections were given to 5 mice in each group (two injection per mouse), and the mice were sacrificed 40 days after injection. Statistical comparison for primary tumors was analyzed by Student's *t test*. Statistical analysis for pooled MRG positive clones relative to pooled MRG negative clones gave  $P < 0.005$  for all the following comparisons: MRG-231-6 vs. MDA-MB-231; vs. neo-231-1; vs. neo-231-2, and MRG-231-10 vs. MDA-MB-231; vs. neo-231-1; and vs. neo-231-2.

10 30 50  
 GGGGAAAGGGCAAGGATGGTGGAGGCTTTCTGTGCTACCTGGAAGCTGACCAACAGTCAG  
 70 90 110  
 AACTTTGATGAGTACATGAAGGCTCTAGGCGTGGGCTTTGCCACTAGGCAGGTGGGAAAT  
 M K A L G V G F A T R Q V G N  
 130 150 170  
 GTGACCAAACCAACGGTAATTATCAGTCAAGAAGGAGACAAAGTGGTCATCAGGACTCTC  
 V T K P T V I I S Q E G D K V V I R T L  
 190 210 230  
 AGCACATTCAAGAACACGGAGATTAGTTTCCAGCTGGGAGAAGAGTTTGATGAAACCACT  
 S T F K N T E I S F Q L G E E F D E T T  
 250 270 290  
 GCAGATGATAGAACTGTAAGTCTGTTGTTAGCCTGGATGGAGACAACTTGTTACATA  
 A D D R N C K S V V S L D G D K L V H I  
 310 330 350  
 CAGAAATGGGATGGCAAAGAAACAAATTTTGTAAAGAGAAATTAAGGATGGCAAATGGTT  
 Q K W D G K E T N F V R E I K D G K M V  
 370 390 410  
 ATGACCCTTACTTTTGGTGATGTGGTTGCTGTTCCGCACTATGAGAAGGCATAAAAATGT  
 M T L T F G D V V A V R H Y E K A \*  
 430 450 470  
 CCCTGGTCGGGGCTTGGAAGAGCTCTTCAGTTTTTCTGTTTCCTCAAGTCTCAGTGCTAT  
 490 510 530  
 CCTATTACAACATGGCTGATCATTAATTAGAAGGTTATCCTTGGTGTGGAGGTGGAAAT  
 550 570 590  
 GGTGATTTAAAACTTGTTACTCCAAGCAACTTGCCCAATTTTAATCTGAAAATTTATCA  
 610 630 650  
 TGTTTTATAATTTGAATTAAAGTTTTGTCCCCCCCCCTTTTTTTTATAAACAAGTGAAT  
 670 690 710  
 ACATTTTATAATTTCTTTTGAATGTAAATCAAATTTGAATAAAAATCTTACACGTGAAA  
 730  
 AAAAAAAAAA

Figure 1

1	MVEAFCATWK	LTNSQNFDEY	MKALGVGFAT	RQVGNVTKPT	MRG
1	MVDAFVGTWK	LVDSKNFDDY	MKSLGVGFAT	RQVGNMTKPT	Bovine MDGI
1	MADAFVGTWK	LVDSKNFDDY	MKSLGVGFAT	RQVASMTKPT	Mouse MDGI
1	MVDAFLGTWK	LVDSKNFDDY	MKSLGVGFAT	RQVASMTKPT	H-FABP hu
1	MCDAFVGTWK	LVSSSENFDDY	MKEVGVGFAT	RKVAGMAKPN	A-FABP hu
41	VIISQEGDKV	VIRTLSTFKN	TEISFQLGEE	FDETTADDRN	MRG
41	TIIEKNGDTV	IIKTQSTFKN	TEISFKLGVE	FDETTADDRK	Bovine MDGI
41	TIIEVNGDTI	TIKTQSTFKN	TEINFQLGIE	FDEVTADDRK	Mouse MDGI
41	TIIEKNGDIL	TLKTHSTFKN	TEISFKLGVE	FDETTADDRK	H-FABP hu
41	MIISVNGDLV	TIRSESTFKN	TEISFKLGVE	FDEITADDRK	A-FABP hu
81	CKSVVSLDGD	KLVHIQKWDG	KETNFVREIK	DGKMVMTLTF	MRG
81	VKSIIVTLDGG	KLVHVQKWNG	QETSLVREMV	DGKLILTLTH	Bovine MDGI
81	VKSLVTLDGG	KLIHVQKWNG	QETTTLRELV	DGKLILTLTH	Mouse MDGI
81	VKSIIVTLDGG	KLVHLQKWDG	QETTTLVRELI	DGKLILTLTH	H-FABP hu
81	VKSIITLDGG	ALVQVQKWDG	KSTTIKRKRD	GDKLVVECVM	A-FABP hu
121	GDVVAVRHYE	KA-			MRG
121	GTAVCTRITYE	KQA			Bovine MDGI
121	GSVVSTRITYE	KEA			Mouse MDGI
121	GTAVCTRITYE	KEA			H-FABP hu
121	KGVTSTRVYE	R-A			A-FABP hu

Figure 2

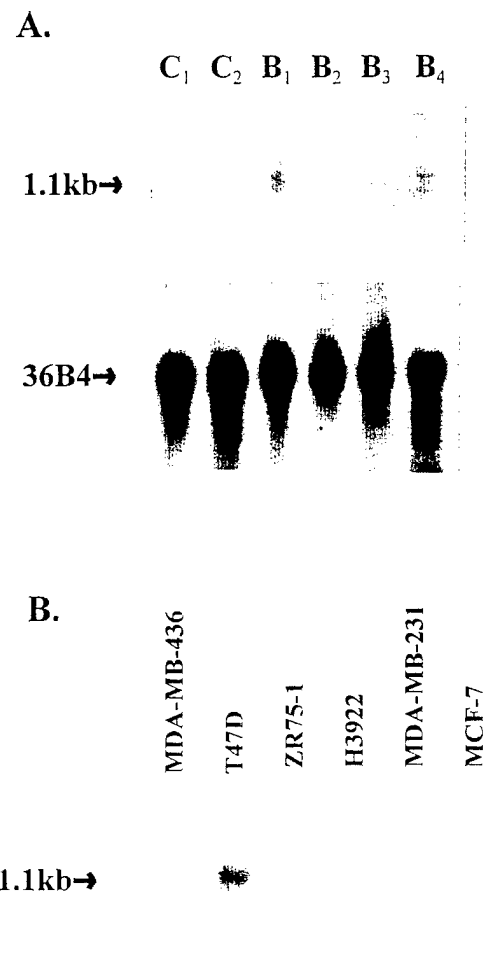


Figure 3

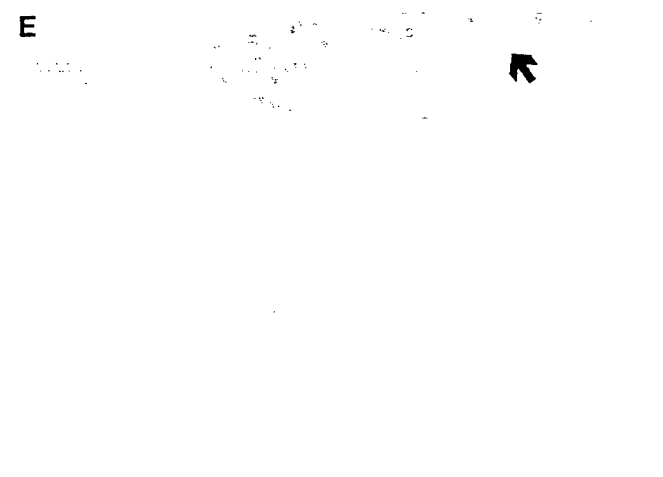
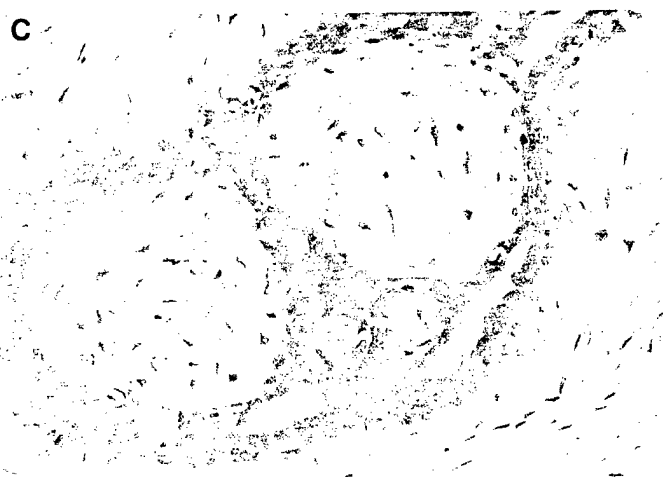
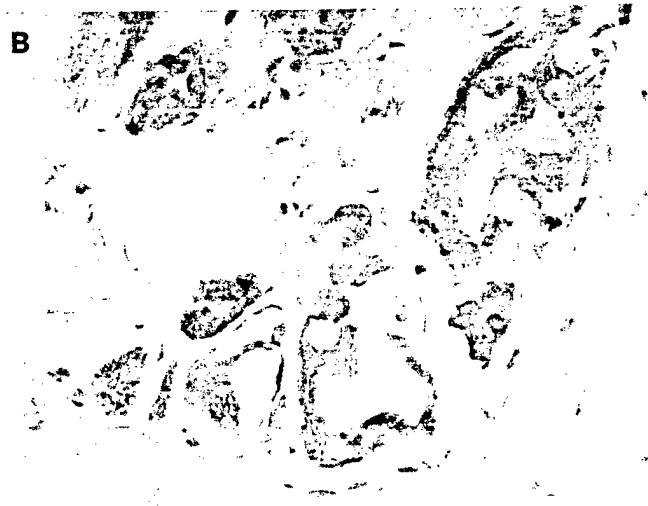
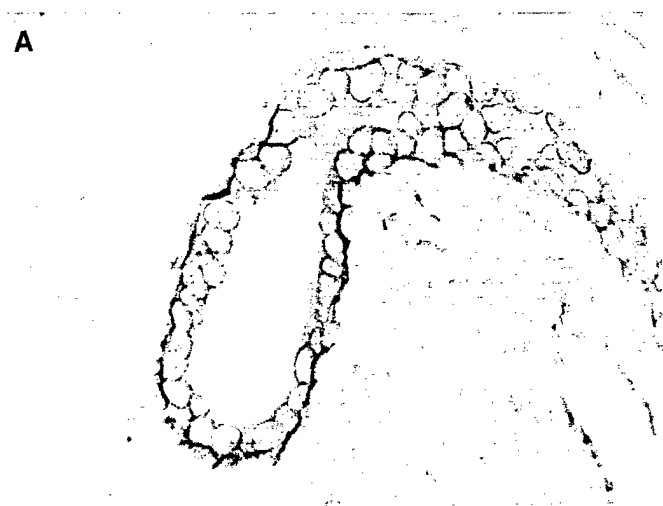


Figure 4



Figure 5

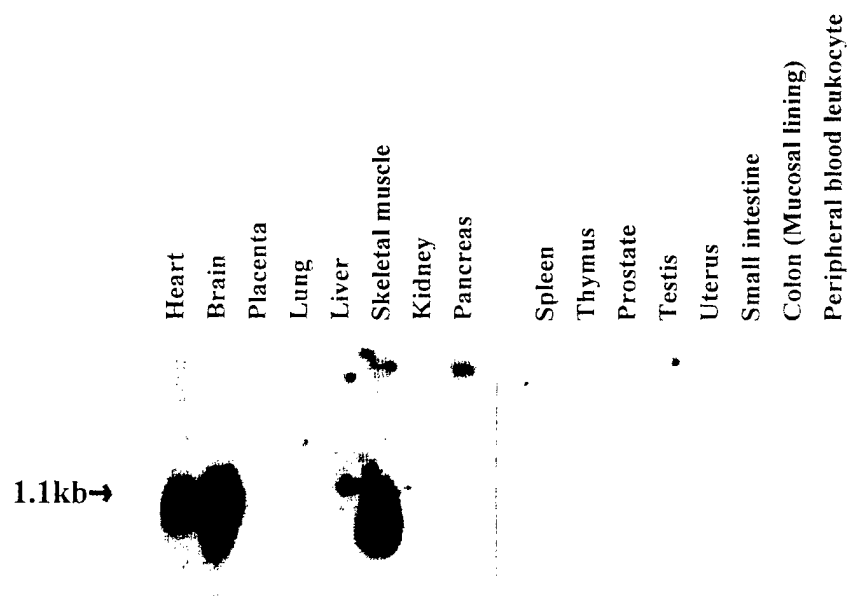


Figure 6

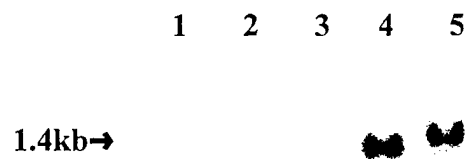


Figure 7

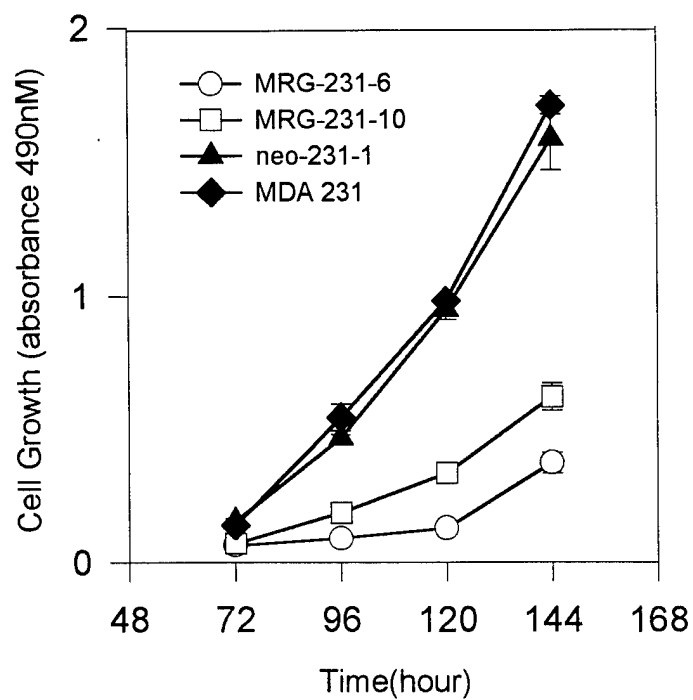


Figure 8

